Mitotic entry and exit: the rise and fall of cyclin-Cdk-Cks activity

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The APC/C recruits cyclin B1-Cdk1-Cks in prometaphase before D-box recognition to control mitotic exit

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Summary

The ubiquitin ligase APC/C is activated at prometaphase by mitotic phosphorylation and binding of its activator Cdc20. This initiates cyclin A degradation while cyclin B1 is stabilized by the spindle checkpoint. Upon checkpoint release, the RXXL destruction motif (D-box) was proposed to direct cyclin B1 to core APC/C or Cdc20. Here, we report that endogenous cyclin B1-Cdk1 is recruited to checkpoint-inhibited, phosphorylated APC/C in prometaphase, independently of Cdc20 or the cyclin B1 D-box. Like cyclin A, cyclin B1 binds the APC/C by the Cdk-cofactor Cks, and the APC3 subunit. Prior binding to APC/C-Cdc20 makes cyclin B1 a better APC/C substrate in metaphase, driving mitotic exit and cytokinesis. We conclude that, in prometaphase, the phosphorylated APC/C can recruit both cyclin A and cyclin B1 in a Cks-dependent manner. This suggests that the spindle checkpoint blocks D-box recognition of APC/C-bound cyclin B1, whereas distinctive complexes between the N-terminus of cyclin A and Cdc20 evade checkpoint control.

Introduction

Ubiquitin-dependent destruction of proteins that inhibit an upcoming event in the cell cycle provides a mechanism to govern unidirectional cell cycle progression. Like in lower organisms, in mammalian cells cyclin B1-Cdk1 is the principal kinase catalyzing entry of G2-phase cells into mitosis (Lindqvist et al., 2009) but cyclin B1 degradation and Cdk1 inactivation drive mitotic exit and cytokinesis (Clute and Pines, 1999;Hagting et al., 2002;Sullivan et al., 2008;Wolf et al., 2006). Destruction of Separase inhibitor Securin at metaphase is essential for sister chromatid separation (Hagting et al., 2002;Yanagida, 2005). In mammalian cells, synchronized loss of cyclin B1 and Securin thus coordinates cell division with nuclear division (Pines, 2006).

By the action of spindle checkpoint proteins (e.g. Mad1, Mad2, Bub1, BubR1, and Mps1), cyclin B1 and Securin are stabilized until all chromosomes are bi-polarly attached to the mitotic spindle at metaphase (Kops, 2008;Sczaniecka and Hardwick, 2008;Kulukian et al., 2009). The spindle checkpoint proteins cooperate to inhibit the function of Cdc20, a WD40-repeat containing protein (Yu, 2007) that binds to and activates the APC/C. APC/C-Cdc20 forms a multi-subunit E3 ubiquitin ligase that directs proteasomal destruction of cyclin B1 and Securin upon release of the spindle checkpoint (van Leuken et al., 2008;Pines, 2006;Yu, 2007).

The spindle checkpoint does not preclude binding of Cdc20 to the APC/C (Sczaniecka and Hardwick, 2008; Nilsson et al., 2008; Kulukian et al., 2009; Herzog et al., 2009). By their destruction region (involving the RXXL D-box), APC/C substrates may interact with the WD40 domains of Cdc20 (Ohtoshi et al., 2000;Hilioti et al., 2001;Kraft et al., 2005; Passmore and Barford, 2005). Checkpoint proteins could on their turn block substrate binding to Cdc20, as shown in vitro, suggesting that the spindle checkpoint prevents recruitment of substrates to APC/C-Cdc20 (Herzog et al., 2009). Spindle-checkpoint proteins can also induce conformational changes in the

APC/C itself, thus repositioning Cdc20 (Herzog et al., 2009). This could mean that release of the spindle checkpoint helps to functionally activate Cdc20. The checkpoint further either promotes poly-ubiquitination and destabilization of Cdc20 (Nilsson et al., 2008;Ge et al., 2009) or inhibits Cdc20 poly-ubiquitination to prevent its activation (Stegmeier et al., 2007). In conflict with the view that the checkpoint must be released before substrates can be recognized or Cdc20 can be activated, is the long-standing observation that APC/C-Cdc20 is already active in prometaphase, targeting cyclin A for destruction while cyclin B1 remains stable (Den Elzen and Pines, 2001;Geley et al., 2001;Stewart et al., 1994;Wolthuis et al., 2008).

This paradox is not explained by inferring that cyclin A is an extremely efficient APC/C substrate, requiring minimal amounts of Cdc20 for its destruction, since partial depletion of Cdc20 by RNAi delays cyclin A destruction at least as well as cyclin B1 destruction (Wolthuis et al., 2008). The C-terminal di-peptide of the prometaphase APC/C-Cdc20 substrate Nek2A acts as a direct APC/C binding motif (Hayes et al., 2006), suggesting that prometaphase APC/C-Cdc20 substrates may be recruited to the APC/C independently of Cdc20 to escape checkpoint control. However, Cdc20 is a rate-limiting factor for both cyclin A and Nek2A destruction (Wolthuis et al., 2008;Hayes et al., 2006;Kulukian et al., 2009). This indicates that, rather, an un-inhibited pool of Cdc20 may specifically direct the destruction of prometaphase APC/C substrates.

To be degraded in prometaphase, cyclin A depends on the conserved Cdk co-factors called Cks (the mammalian p9 proteins Cks1 and Cks2, orthologous to fission yeast Suc1 and Xenopus Xe-p9, here collectively referred to as Cks). Cks can bind phosphorylated cyclin-Cdk substrates, such as the APC/C, by its anion binding pocket (Patra et al., 1999;Pines, 1996;Shteinberg and Hershko, 1999;Sudakin et al., 1997). This suggests that Cks functions to stabilize complexes between cyclin-Cdks and their phosphorylated ligands. It could be acting as a processivity factor to facilitate multi-phosphorylation events by cyclin-Cdks that are retained to their pre-phosphorylated substrates (Pines, 1996). The Dunphy lab showed that Cks-dependent phosphorylation by cyclin B1-Cdk1 is required to activate the Xenopus APC/C in mitotic extracts (Patra and Dunphy, 1998). However, it is also possible that Cks acts as a targeting subunit, recruiting cyclin A to the APC/C when the APC/C is phosphorylated in mitosis (Wolthuis et al., 2008).

Apart from cyclin A, cyclin B1 binds to Cks, too. Here we investigated interplay between cyclin B1-Cdk-Cks and the mitotic APC/C. We found that whereas the sub-cellular localization of cyclin B1-Cdk1 was independent of Cks, Cks promoted mitotic APC/C phosphorylation and activation, as measured by Securin destruction in cells. Remarkably, Cks also retained cyclin B1-Cdk1 to spindle checkpoint-inhibited APC/C-Cdc20 as a substrate, secondary to enhancing APC/C phosphorylation. This required APC3, but not Cdc20 or the cyclin B1 D-box, yet enhanced cyclin B1 turn-over in metaphase to protect cells against cytokinesis failure.

We conclude that Cks plays a dual role in cyclin B1 destruction in human cells: to activate the APC/C by promoting cyclin B1-Cdk1-dependent phosphorylation, and

to facilitate binding of cyclin B1 to phosphorylated APC/C-Cdc20 to make it a better substrate. Our data imply that cyclin B1 is recruited to APC/C-Cdc20 well before recognition of the D-box by Cdc20 as triggered by release of the spindle checkpoint (Kraft et al., 2005;Herzog, et al. 2009). In prometaphase, cyclin B1, like cyclin A, is directed to phosphorylated APC/C by its Cdk and Cks partners, but cyclin A differs from cyclin B1 in the way its N-terminal destruction region binds to Cdc20 in G2 phase. This may facilitate competition between cyclin A and spindle checkpoint proteins in mitosis. We conclude that processive mitotic cyclin ubiquitination requires their Cks-dependent retention at the APC/C in the spindle checkpoint, a binding step which precedes D-box dependent ubiquitination.

Results

Cks proteins facilitate cyclin B1-Cdk1-dependent APC/C phosphorylation and activation

We aimed to study the roles of both Cks1 and Cks2 downstream of cyclin B1-Cdk1. Therefore, we used a non-Cks binding mutant of human Cdk1, Cdk1-P242L. We call this mutant Cdk1-1N as it is orthologous to budding yeast *Cdc28-1N* that has a normal G1 and S function but confers a mitotic defect (Surana et al., 1991). To follow how mitotic progression depends on the ability of Cdk1 to bind Cks, we aimed to complement cells depleted of endogenous Cdk1 by fluorescent Cdk1 or Cdk1-1N constructs.

We had found that Cdk1-EYFP, when over-expressed in control cells, does not localize like cyclin B1 in mitotic cells (Fig. 1A, Supplemental Fig. 1A). However, this was different in cells from which endogenous Cdk1 was depleted, and RNAi-resistant Cdk1-EYFP (Cdk1*-EFYP) was used to restore expression (Fig. 1C; transient co-transfections in U2OS cells). In these cells, fluorescent Cdk1 co-localized with cyclin B1 on centrosomes in G2 phase, entered the nucleus in prophase and stained mitotic spindle, chromosomes and kinetochores in prometaphase (Fig. 1C; Supplemental Fig. 1B). At metaphase, Cdk1 localization became dispersed as cyclin B1 was degraded (Fig. 1C, 'M'). Cdk1 remarkably escaped protein destruction, probably by dissociating from cyclin B1 at the proteasome (Nishiyama et al., 2000; Chesnel et al., 2007). Whereas Cdk1-depleted cells show mitotic defects, restoring expression by RNAi-resistant, fluorescent Cdk1 completely restored normal mitotic progression (Fig 1C,E) (Lindqvist et al., 2007). We conclude that fluorescently-tagged Cdk1 is functional, and also that the intracellular localization pattern of fluorescent, RNAi-resistant Cdk1 acts as a single-cell read-out for efficient depletion of endogenous Cdk1 by our shCdk1 plasmid.

Cyclin B1-binding directs Cdk1 localization (Pines and Hunter, 1994;Draviam et al., 2001;Bentley et al., 2007). Normally, Cdk1 is present in surplus over cyclin B1 (Arooz et al., 2000), but in cells from which endogenous Cdk1 is depleted, increased numbers of cyclin B1-Cdk1-EYFP complexes can be formed. This strongly indicates that the distinct cyclin B1-like localization pattern of Cdk1-*EFYP as shown in Figure 1C is also a marker for binding of Cdk1-*EYFP to cyclin B1.

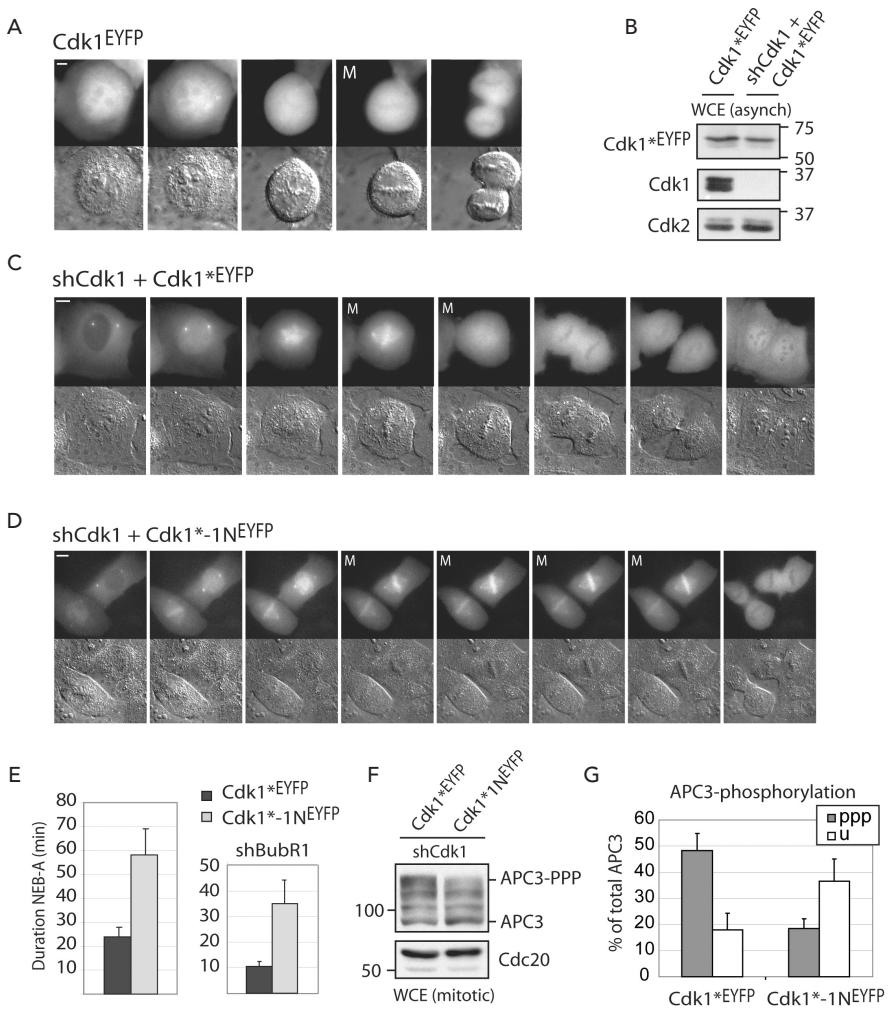


Figure 1. Cdk1-bound Cks proteins enhance APC/C phosphorylation and mitotic progression. (A) Cdk1-EYFP shows no distinct intracellular localization. M denotes metaphase alignment and onset of cyclin B1 destruction in U2OS cells. Scale bar is 5μM. (B) Complementation of Cdk1 RNAi by RNAi-resistant Cdk1*-EYFP. (C) Reconstitution of Cdk1-depletion by Cdk1*-EYFP reveals cyclin B1-binding; see Supplemental Fig 1B. Cdk1 localization gets dispersed upon cyclin B1 degradation at metaphase (M). (D) Complementation of Cdk1 depletion by non-Cks binding Cdk1*-1N delays cells in mitosis. C, D: scale bar is 10μM. (E) Reconstituted expression of fluorescent wild-type Cdk1 rescues Cdk1-RNAi (Lindqvist et al., 2007); NEB to anaphase (NEB-A) is normal, approximately 24 min (n=8). Cdk1*-1N-EYFP complemented cells are delayed in mitosis, NEB-A is 57 min (n=7). The right graph shows Cdk1*-1N-EYFP complemented cells delay independently of the spindle checkpoint (n=7; errors are standard deviations). Knock-down of BubR1 (shBubR1, (Lens et al., 2003) was verified by staining imaged cells with anti-BubR1 after fixation (not shown). (F) Mitotic cells arrested in the spindle checkpoint were collected. Extracts were blotted with indicated antibodies. (G) Intensities of the most phosphorylated forms of APC3, and the unphosphorylated APC3, from four different experiments as in F), were quantified. Errors are standard deviations. A detailed description is shown in Supplemental Figure 1E.

To investigate the function of Cks binding to Cdk1, we used our Cdk1-complementation assay and followed G2 and mitosis in fluorescent cells. Cdk1*-1N-EYFP expressed in Cdk1-depleted cells revealed a similar intracellular localization as wild-type fluorescent Cdk1, with only modest reduction of spindle pole-staining in some cells (Fig. 1D). A potential role for Cks in controlling the G2/M transition was found in Xenopus (Patra and Dunphy, 1996), but as far as we could detect, Cdk1*-1N U2OS cells entered mitosis with normal kinetics. However, Cks-depleted cells delayed in mitosis, most likely in metaphase (Fig. 1D, 1E, left bar chart; Supplemental Fig 1D; Wolthuis et al., 2008) while Cks binding did not affect cyclin-associated Histone 1 kinase activity in vitro (Wolthuis et al., 2008).

A Cks-dependent delay in mitotic exit was not rescued by inactivation of the spindle checkpoint (Fig. 1E, right bar chart). This shows that non-Cks binding Cdk1 delays mitotic exit independently or downstream of the checkpoint. Analysis of APC3 mobility shifts on blots confirmed that the Cdk1-Cks connection supports maximal APC/C phosphorylation in human cells (Fig. 1F; Fig. 1G; Supplemental Fig. 1C and E; Wolthuis et al., 2008). Both Cdk1* and Cdk1*-1N were equally stable in metaphase and anaphase (Supplemental Fig. 1D). We conclude from these experiments that, in human cells, Cks does not control cyclin B1-Cdk1 localization but Cdk1-dependent APC/C phosphorylation and metaphase progression.

Cks proteins promote metaphase APC/C activity, supporting cytokinesis

Cks1 and Cks2 fulfill overlapping roles in mitosis (Wolthuis et al., 2008;Donovan and Reed, 2003). Depletion of 75-85% of both Cks1 and Cks2 by RNAi (further referred to as Cks depletion) did not reduce protein levels of Cdc20, cyclin A or cyclin B1 in U2OS cells (Wolthuis et al., 2008), in contrast to more severe effects of knocking-out Cks1 and Cks2 (Martinsson-Ahlzen et al., 2008). To investigate if Cks depletion affected metaphase APC/C activity in human cells, like in Xenopus extracts (Patra and Dunphy, 1998), we quantified destruction of a fluorescent version of the APC/C-Cdc20 substrate Securin (Securin-Venus). Cells were treated either with siRNA oligos targeting Cks1 alone (as a negative control), or by a combination of siRNAs targeting Cks1 and Cks2. Depleting a single type of Cks had no effect on Securin destruction or cyclin B1 destruction, starting roughly 20 min after nuclear envelope breakdown (NEB) (Fig. 2A,B, siCks1 panels; for further siRNA controls, see (Wolthuis et al., 2008)). In cells depleted of both Cks1 and Cks2, metaphase onset was delayed, probably due to cyclin A stabilization, but Securin destruction started upon chromosome alignment, at metaphase (Fig. 2A, panel labeled 'siCks1 and Cks2'). However, the time required to destroy similar levels of fluorescent Securin was increased nearly two-fold (Fig. 2A, 2C, black and white triangles; Supplemental Fig. 2A).

Normally degradation of cyclin B1 and Securin always occurs simultaneously (Hagting et al., 2002, also see Fig. 7A, middle panel), but, remarkably, in Cks-depleted cells destruction of cyclin B1 was delayed as compared to Securin destruction (Fig. 2B, quantitated in Fig. 2C and Supplemental Fig. 2A). In up to 50% of the cells expressing

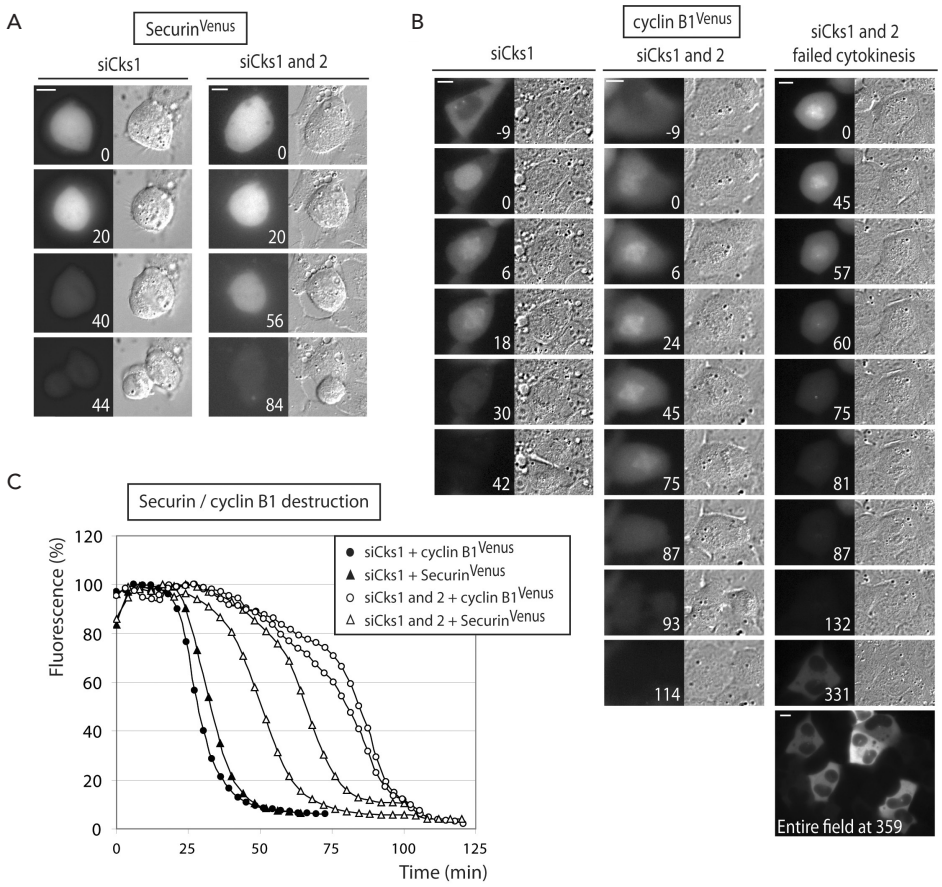


Figure 2. Cks promotes APC/C–dependent destruction of cyclin B1 and Securin. (A,B) Cells were treated with siRNA-pools targeting Cks1 (siCks1, control) or both Cks1 and Cks2 (siCks1 and 2). Total amounts of siRNA were kept equal. Cells transfected with either (A) Securin-Venus or (B) cyclin B1-Venus and expressing similar fluorescent protein levels were followed during mitosis. See Supplemental Fig. 2A for quantified fluorescence levels and total destruction times. (C) Fluorescence levels plotted over time, starting 3 minutes before NEB. Data are representative of 3 independent experiments for each condition. All scale bars are 10μM.

fluorescent cyclin B1, this resulted in delayed or impaired cleavage furrow ingression, or a full cytokinesis-defect (Fig. 2B, right panels; bottom image shows accumulation of undivided cells). Although this phenotype was seen in cells expressing ectopic cyclin B1, control cells degraded at least 5-fold higher amounts of ectopic cyclin B1 and successfully completed cytokinesis (Supplemental Fig. 2A, middle bars). This suggested that Cks proteins do not just control general metaphase activity of the APC/C but, at least in human cells, may have an additional, specific role in cyclin B1 destruction that helps to protect against cytokinesis failure.

Mitotic APC/C phosphorylation was similarly impaired after Cks-depletion (Fig. 3A) as upon reconstituting Cdk1 by non-Cks-binding Cdk1 (Fig. 1F and G, Supplemental Fig. 1E). Interestingly, partial Cks depletion by RNAi clearly reduced formation of stabilized interactions between APC/C-Cdc20 and endogenous cyclin B1-Cdk1 in mitosis (Fig. 3A,B and C). Binding was reduced further when mitotic Cks RNAi cells were also depleted of the APC/C kinase Plk1, which clearly suggests that the APC/C binds cyclin B1 in a way that depends on APC/C phosphorylation (Fig. 3B and C).

Cks retains cyclin B1 at the APC/C as an APC/C substrate

Could Cks-mediated binding of cyclin B1-Cdk1 to the APC/C make cyclin B1 a better substrate for the APC/C? To test this in cells, we aimed to measure destruction of cyclin B1 molecules bound to non-Cks-binding Cdk1-1N. In this assay, we would be able to address if fluorescent cyclin B1 would become a less efficient APC/C-Cdc20 substrate simply by no longer being able to bind Cks. Any effect found would depend exclusively on a role for Cks in targeting cyclin B1 to the APC/C, because endogenous

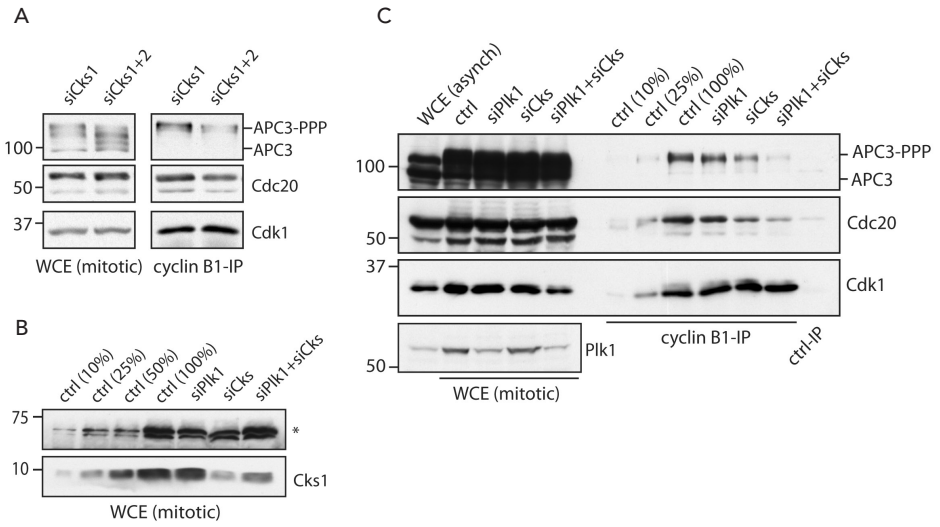


Figure 3. Cks proteins direct cyclin B1 binding to the phosphorylated APC/C. (A) Cells treated as in Fig 2A). The cells were Thymidine released, blocked in Nocodazole and collected by mitotic shake-off. Extracts were blotted with indicated antibodies. IPs with anti-cyclin B1 antibodies were performed and blotted to examine co-precipitation of indicated proteins. APC3 binding in cyclin B1 IPs correlates with the hyper-phosphorylated APC3 (APC3-PPP) present in extracts. (B) Cells were treated with siRNA pools targeting Plk1 (siPlk1), both Cks1 and Cks2 (siCks), or Plk1, Cks1 and Cks2 (siPlk1+siCks). Mitotic cells were collected as in A) and extracts Western blotted and probed with anti-Cks1 antibody. The top panel shows a background band detected by the Cks antiserum, indicated by an asterisk. Serial dilutions of control mitotic cells show Cks1 knockdown is approximately 75% after siCks or between 50 and 75% after siPlk1+siCks. The asterisk at the top panel indicates a background band. (C) The same lysates shown in B) were used to IP cyclin B1 and blotted to examine APC/C and Cdc20 binding. Serial dilutions of control cyclin B1-IPs are shown as a reference.

Cks levels, and thus APC/C phosphorylation, should remain unchanged by the expression of these reporters.

When we co-expressed cyan fluorescent cyclin B1 with yellow fluorescent Cdk1, both proteins co-localized up to metaphase, even in the presence of endogenous Cdk1. This shows that fluorescent Cdk1, which alone is dispersed in the cytoplasm (Fig. 1A), is often complexed to fluorescent cyclin B1 when these proteins are co-expressed at the right levels (Fig. 1,4A). Thus, by analyzing cells in which fluorescent cyclin B1

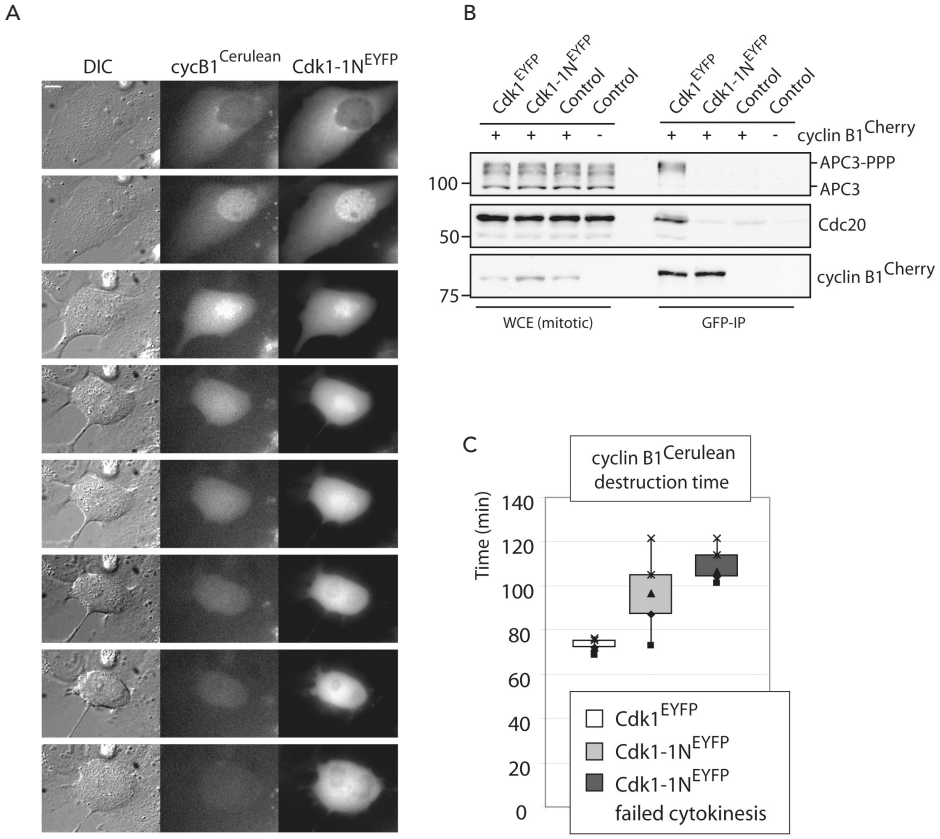


Figure 4. Cks promotes recruitment of cyclin B1 as an APC/C substrate. (A) Cells were co-transfected with cyclin B1-Cerulean and either Cdk1*-EYFP or Cdk1*-1N-EYFP. Shown is a cell undergoing mitotic exit without cytokinesis. Scale bar is 5μM. (B) Reduced APC/C-Cdc20 binding of cyclin B1-Cdk1-1N complexes in cells, while total APC/C phosphorylation is normal. Checkpoint-arrested mitotic cells, selected for expression of indicated constructs, were collected. GFP-IPs (pulling down Cdk1-EYFP) on equalized extracts were blotted for co-precipitation of indicated proteins. Cyclin B1-Cherry is not recognized by the anti-GFP antibodies. (C) Also see Supplemental Fig. 2B. Quantitative comparison of destruction times of cyclin B1, bound to Cdk1 (white box), or to Cdk1-1N (light gray box). The destruction times of the cells expressing Cdk1-1N which failed cytokinesis are presented in the dark gray box. The plots contain data from 3 independent experiments (see Supplemental Fig 2B for destruction curve).

co-localized with fluorescent Cdk1, indicative of their complex formation, we could address if binding of cyclin B1 to Cks could make it a better APC/C-Cdc20 substrate in cells. To ensure that ectopic expression did not affect normal cell division, only the cells that showed correct onset of cyclin B1 destruction at chromosome alignment, or Cdk1-mutant cells showing highly comparable cyclin B1 fluorescence levels as control, were analyzed.

Interestingly, metaphase degradation of ectopic, fluorescent cyclin B1 bound to fluorescent Cdk1-1N was again significantly delayed as compared to normal fluorescent Cdk1 control (Fig. 4C, and Supplemental Fig. 2B). Cytokinesis failed in 50% of these cells, too (Fig. 4A and C). Crucially, total mitotic APC/C phosphorylation was unchanged, but cyclin B1-Cdk1-1N complexes still failed to bind phosphorylated APC/C (Fig. 4B). A model emerges from these findings in which cyclin B1-Cdk1 needs to bind Cks to stay bound to, or to flux onto, phosphorylated APC/C in prometaphase. This would direct efficient processing of cyclin B1 as an APC/C substrate in metaphase, control mitotic exit and safeguard cytokinesis.

Complex formation between cyclin B1 and phosphorylated APC/C-Cdc20 in mitosis

To study when endogenous cyclin B1 binds to the APC/C and Cdc20, we analyzed their cell-cycle dependent interactions. Cyclin B1, immuno-precipitated from extracts of mitotic cells, arrested in Nocodazole and collected by mitotic shake-off (95% 4N, >90% positive for MPM2 as analyzed by FACS, see Methods), was bound to APC/C-Cdc20. This interaction was not found in extracts of G2-phase cells (Fig. 5A,B, Supplemental Fig. 3A,3D). In contrast, we found few complexes between APC/C or Cdc20 and Securin in G2 phase or mitosis (either with endogenous or ectopically expressed, tagged versions) (Supplemental Fig. 3B,C).

Cyclin B1 bound exclusively to the maximally phosphorylated APC/C fraction (APC3-PPP, Fig. 5A, compare APC3 input, lower panel, to APC3 captured in the cyclin B1 IP, top panel). Furthermore, the APC/C retained in cyclin B1 immuno-precipitations (kept in mitosis by proteasome inhibition after inactivation of the spindle checkpoint) was functionally active in vitro (Fig. 5C) in a way dependent on ATP, E1, and APC/C-specific E2 enzyme (Supplemental Fig. 3E,F,G and unpublished data). Mitosis-specific interactions between APC/C-Cdc20 and cyclin B1 were further found in APC2, APC3, APC4, APC6, Cdc20, cyclin B1 and Cdk1 IPs (Supplemental Fig. 3D and unpublished data). We estimated that more than 25% of endogenous cyclin B1 was bound to the mitotic APC/C in Nocodazole-arrested cells (see Materials and Methods and further evidence in Fig. 8B). Similar complexes were found in extracts of cells collected by mitotic shake-off 12 hrs after Thymidine release in the absence of any spindle drugs (Supplemental Fig. 3A), in mitotic cells arrested by Nododazole or Taxol (Fig. 5D and unpublished data), or in cells released from the spindle checkpoint but kept in mitosis by proteasome inhibition (Fig. 5D, 'NR- MG132'). In conclusion, a significant fraction of endogenous cyclin B1 is recruited to proper APC/C and Cdc20 in prometaphase, regardless of the cyclin B1-stabilizing effect of the mitotic spindle checkpoint.

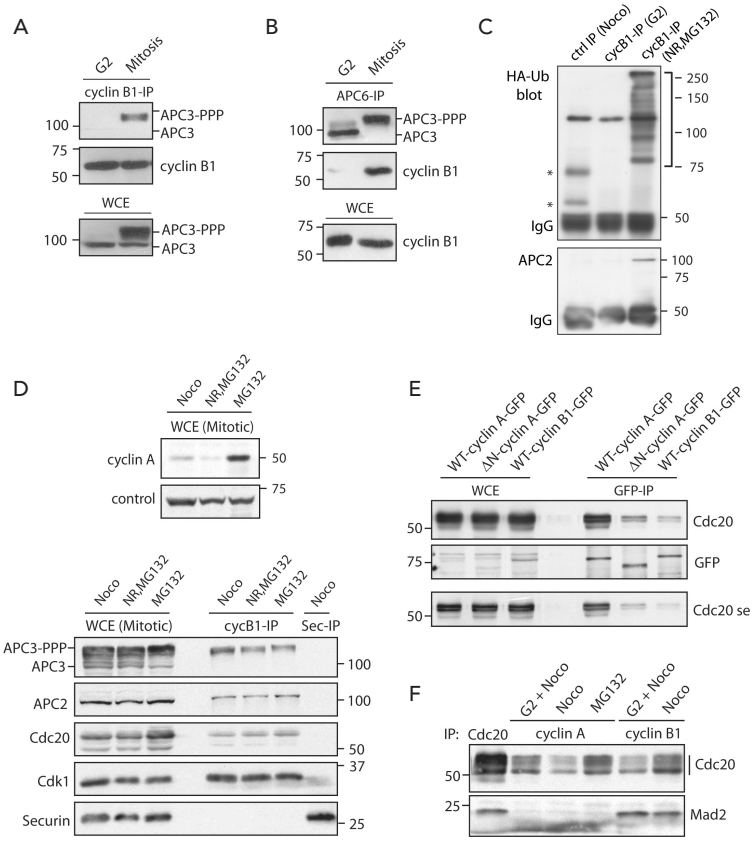


Figure 5. Binding of phosphorylated APC/C-Cdc20 to cyclin B1 starts in mitosis. (A) Cyclin B1-IPs from synchronized G2-phase (G2) and Nocodazole-arrested mitotic cells (Mitosis) are shown. For synchronization details see Materials and Methods. IPs were analyzed for (co-)precipitating proteins by Western, probed for anti-APC3 (top panel, showing only phosphorylated APC3) or anti-cyclin B1 (middle panel). Lower panel shows the presence of unphosphorylated (lane 1) as well as phosphorylated APC3 (lane 2) in extracts (WCE) used for the IPs (10% of input). (B) APC/C-IPs using anti-APC6 antibodies on extracts of G2-phase cells and mitotic cells. (C) Cyclin B1 IPs on extracts from G2 or mitotic cells were used in in vitro ubiquitination assays. Mitotic cells arrested in Nocodozole were released but kept in mitosis by the proteasome inhibitor MG132 (NR, MG132). Control: GFP-IPs. Purified E1 enzyme, the APC/C specific E2 enzyme UbcH10, ATP and HA-ubiquitin were added to start the reactions. HA-ubiquitin conjugated reaction products were probed with anti-HA antibody. For further controls see Supplemental Fig.3. (D) Comparison of mitotic cells arrested in the spindle checkpoint (Noco), released from the spindle checkpoint arrest but kept in mitosis by MG132 (NR, MG132), or allowed to enter mitosis in the presence of MG132 (MG132); the latter contain high cyclin A levels (upper panels). Cyclin B1 IPs are shown; negative control: Securin (Sec) IP. Western blots were analyzed for (co-)precipitating proteins with indicated antibodies. Top panel shows phosphorylated forms of APC3 in cyclin B1 IPs. (E) Cells were transfected and selected for expression of indicated cyclin-GFP fusion proteins. Mitotic cells that had entered mitosis in the presence of the proteasome inhibitor MG132 were collected by shake-off. GFP-IPs were probed for co-precipitation of Cdc20. (F) Cells were synchronized in G2 and mitosis as in indicated. Anti-cyclin AIPs, anti-cyclin B1 IPs and mitotic extracts were blotted for APC3, Cdc20 and Mad2. Comparable polyclonal antibodies were used, detecting the N-terminal 430 amino-acids of the respective cyclins.

Cyclin A and cyclin B1 bind to different pools of Cdc20

Our previous work indicated a key role for Cks in directing cyclin A-Cdk complexes to the APC/C to promote cyclin A destruction, which evades inhibition by the spindle checkpoint (Wolthuis et al., 2008). Here, we find that Cks recruits cyclin B1 to the phosphorylated APC/C, irrespective of the spindle checkpoint and prior to onset of cyclin B1 destruction. This shows that cyclin A destruction cannot escape spindle checkpoint control simply because it is directed to the APC/C by Cks. Which differences in the way cyclin A and cyclin B1 bind APC/C or Cdc20 may explain their different destruction patterns?

By collecting mitotic cells in the presence of the proteasome inhibitor MG132, we found that binding of cyclin B1 to Cdc20 and phosphorylated APC/C was unaffected by the presence of stabilized cyclin A (Fig. 5D), indicating Cdc20 levels are sufficiently high to bind both cyclins effectively. However, we found a remarkably different timing of complex formation: a significant fraction of cyclin A interacted with Cdc20 in G2 phase, when cyclin B1 bound little or no Cdc20 (Supplemental Fig. 3D; compare input to IP; also see Wolthuis et al., 2008). Importantly, these cyclin A-Cdc20 complexes did not bind to the APC/C (Wolthuis et al., 2008). To compare how cyclin A and cyclin B1 may differ in the way they bind to Cdc20, we expressed GFP-tagged versions of cyclin B1, cyclin A, or an N-terminal cyclin A deletion mutant, arrested transfected cells in mitosis by proteasome inhibition, and determined Cdc20 binding to GFP-IPs. Fig. 5E shows that cyclin A binds much more efficiently to Cdc20 as compared to cyclin B1. Importantly, this difference relates to a feature within the N-terminus of cyclin A that is also required for the spindle checkpoint-independent destruction of cyclin A (Den Elzen and Pines, 2001;Geley et al., 2001).

How could this variation explain the different times at which cyclin A and cyclin B1 are degraded? Cyclin A binds directly to Cdc20 by its N-terminus (Ohtoshi et aL., 2000), even without binding to the APC/C (Wolthuis et al., 2008). This suggests a model in which Cdc20 that is bound to cyclin A escapes inhibition by spindle checkpoint proteins. Recruitment of cyclin A-Cdc20 complexes to the APC/C would depend on Cks and APC/C phosphorylation, which occurs at the same time as cyclin A destruction starts, in prometaphase. In a comparison of cyclin A and cyclin B1 IPs that were normalized for the amount of Cdc20 co-immunoprecipitated, indeed the amount of Mad2 that was detected in cyclin A IPs was much lower as compared to the amount of Mad2 detected in cyclin B1 IPs (Fig. 5F). We suggest from these experiments that cyclin A can bind Cdc20 in a way that prevents efficient inhibition of Cdc20 by the checkpoint.

Altogether, we propose that Cks-binding does not influence responsiveness to the checkpoint, but only the ability of mitotic cyclins to bind the APC/C. This is in agreement with our results that recruitment cyclin B1 to the phosphorylated APC/C depends on Cks and is not blocked by the spindle checkpoint.

Complexes between cyclin B1 and APC/C-Cdc20 depend on APC3, not Cdc20

Our model predicts that, in contrast to cyclin A, cyclin B1 does not bind directly to Cdc20, or much less efficiently. Detection of Cdc20 in cyclin B1-IPs would thus predominantly reflect binding of cyclin B1 to mitotic APC/C-Cdc20 complexes. Because we found that binding of cyclin B1 to the APC/C depends on Cks, and Cks can bind to phosphorylated APC/C in vitro, we tested whether the heavily phosphorylated APC subunit APC3 (Cdc27) could be involved in recruiting cyclin B1 to the APC/C. Therefore, we depleted APC3 to less than 5% of its endogenous levels, leading to a robust mitotic arrest (Supplemental Fig. 4A,B). Cells that arrested in mitosis by APC3 depletion, purified by mitotic shake-off, expressed high levels of cyclin A and cyclin B1, whereas Securin was stabilized partially (Fig. 6A, Supplemental Fig. 5A).

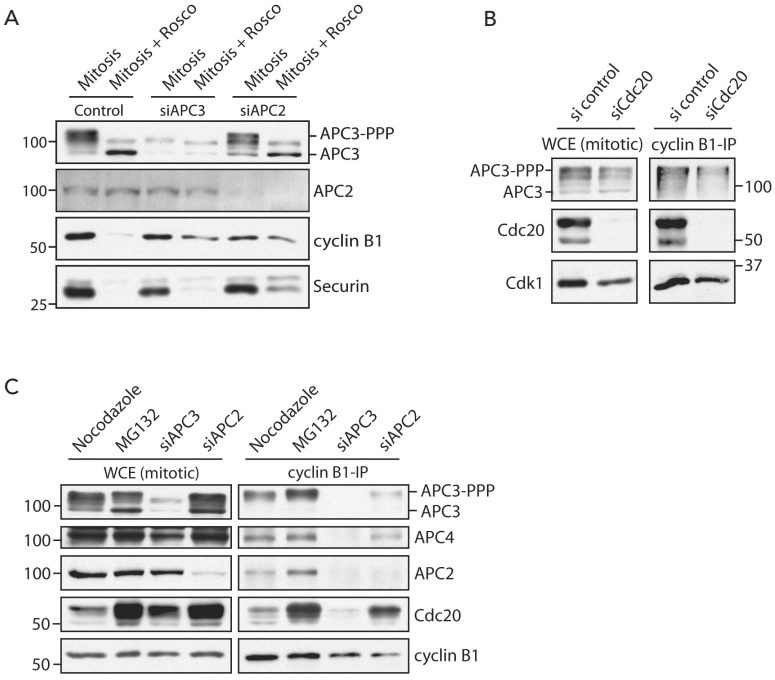


Figure 6. Retention and destruction of Cyclin B1 critically depends on APC3. (A) Cells were arrested in mitosis by Nocodazole after treatment with control siRNA (lanes 1,2) or arrested by treatment of siRNA-pools targeting APC3 (lanes 3,4) or APC2 (lane 5,6). 50% of these mitotic cells were released out of mitosis by the Cdk1 inhibitor Roscovitine (lanes 2, 4 and 6). Extracts were blotted and probed with antibodies against indicated proteins. (B) IPs with anti-cyclin B1 antibodies on extracts of mitotic cells, arrested in the spindle checkpoint (Nocodazole), or arrested by allowing the cells to enter mitosis in the presence of MG132 (MG132), or arrested by treatment of siRNA-pools targeting APC3 or APC2. Extracts and IPs were probed with antibodies against indicated proteins. (C) IPs with cyclin B1 antibodies on extracts of mitotic cells, arrested by the spindle checkpoint and treated with control siRNA or a siRNA-pool targeting Cdc20. IPs were blotted and probed with antibodies against indicated proteins. Identical results were obtained by using short hairpins (shRNA) to target APC3 (Supplemental Fig. 5).

First, we analyzed why Securin was stabilized only partially in APC3 depleted cells. In yeast, over-expression of the Cdk-inhibitor Sic1 induces mitotic exit independently of the APC3 subunit (Thornton and Toczyski, 2003). Here we added the Cdk-inhibitor Roscovitine to mitotic cells, either arrested by spindle drugs, or by depletion of either APC3 or APC2, respectively, to test first if cyclin B1 stabilization depended on the mitotic arrest. These cells initiated cleavage furrow formation and exited mitosis (Supplemental Fig. 4C,D). After inducing mitotic exit in cells arrested by APC3-depletion, cyclin B1 remained largely stable whereas Securin was partially degraded (Fig. 6A, Supplemental Fig. 5B,C). However, Securin was more stable after treatment of these cells with proteasome inhibitor MG132 (Supplemental Fig. 5B), or after depletion of APC2, a cullin-like subunit essential for APC/C ubiquitin ligase activity (Fig. 6A, Supplemental Fig. 5C). This suggests that the APC/C can partially process Securin in the absence of APC3. In contrast, cyclin B1 was equally stabilized after inducing mitotic exit in cells depleted of APC3 or APC2 (Fig. 6A, Supplemental Fig. 5C). Specific securin loss in APC3-depleted mitotic cells may depend on residual APC/C activity, processing Securin more effectively as compared to cyclin B1, in line with Securin being a better APC/C substrate in vitro (Rape et al., 2006). Alternatively, destruction of mitotic cyclins depends more critically on APC3 than destruction of Securin does, like in yeast (Thornton and Toczyski, 2003). Regardless the mechanism of Securin loss, we conclude that APC3 is critical for cyclin B1 destruction, independently of the mitotic arrest.

Next, we investigated the role of APC3 in complex-formation between cyclin B1 and APC/C-Cdc20. In cells depleted of 90-95% of APC3, Cdc20-binding APC/C complexes remained, as in budding yeast and in agreement with the distinct positions of APC3 and Cdc20 in the modeled APC/C structure (Supplemental Fig. 5E,F; Herzog et al., 2009). However, in the absence of APC3, the mitotic APC/C no longer bound cyclin B1 (Fig. 6B, Supplemental Fig. 5D). Depletion of APC2 did not abrogate binding of cyclin B1 to the remaining APC/C-Cdc20, indicating that complex formation between cyclin B1 and APC/C-Cdc20 requires APC3 but not APC2 or APC/C activity (Fig. 6B, compare lanes 3 and 4 of the right panel). Interestingly however, the mitotic APC/C bound normally to cyclin B1 after Cdc20 was completely depleted from cells (Fig. 6C; note the slightly reduced APC3 signal in lane 4 correlates with lower input of cyclin B1 in the IP). Altogether, our findings suggest that cyclin B1 recruitment to APC/C-Cdc20 depends on phosphorylated APC3, whereas Cdc20 is not involved in this step.

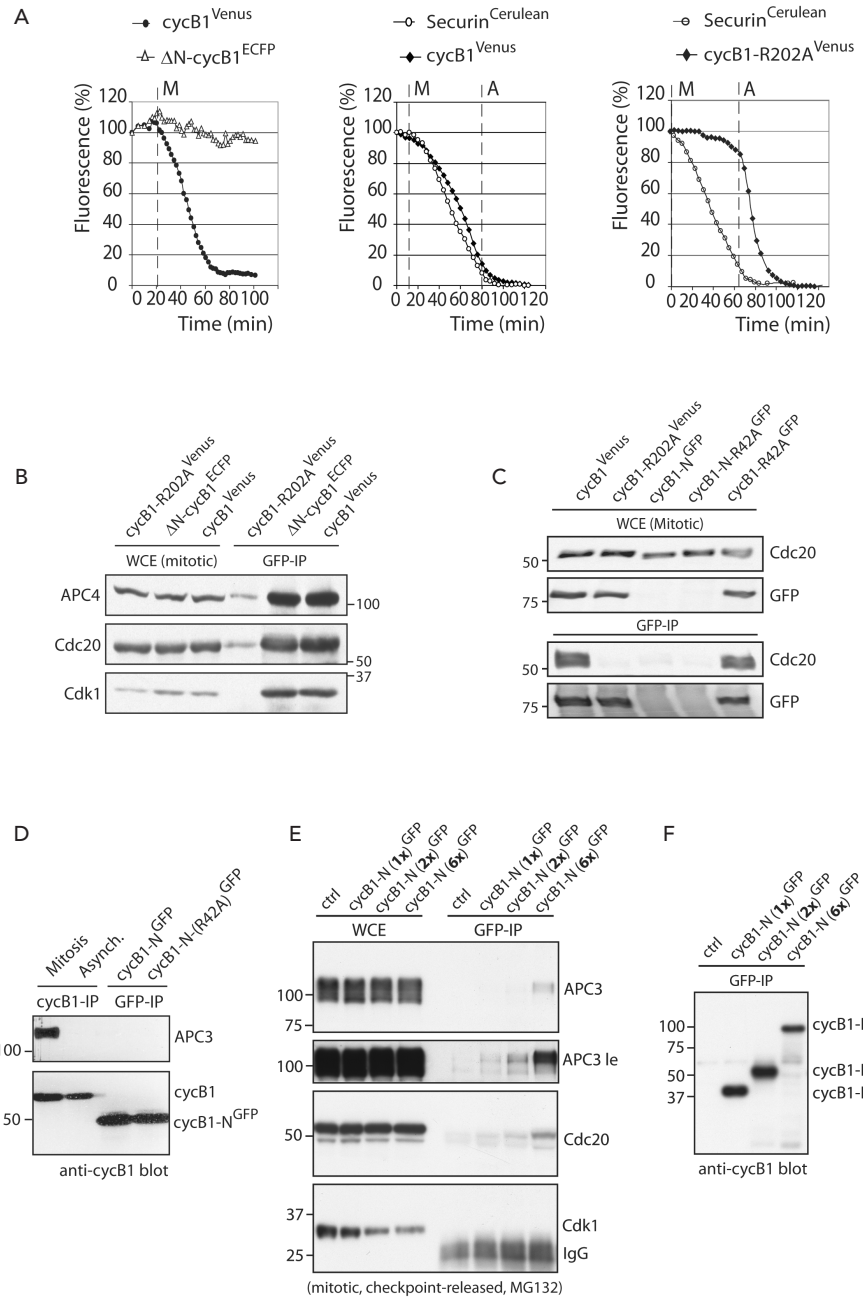
APC/C-Cdc20 binding to cyclin B1 correlates with a substrate-retention step

How does Cks-dependent binding of cyclin B1-Cdk1 to the APC/C contribute to D-box dependent cyclin B1 destruction? Deletion of the N-terminal destruction region or the D-box renders cyclin B1 almost completely stable and blocks cells in mitosis, while co-expressed fluorescent cyclin B1 is degraded because these cells arrest downstream of the spindle checkpoint (Fig. 7A, left panel). This confirms that the D-box is essential for cyclin B1 destruction and cytokinesis. However, a cyclin B1 mutant with a normal

N-terminus yet defective in Cdk1-Cks binding (cycB1-R202A-Venus) also showed an impaired destruction pattern in metaphase, whereas in control cells Securin and cyclin B1 are degraded with similar efficiencies (Fig. 7A, compare middle and right panels). The cyclin B1 R202A mutant was defective for APC/C-Cdc20 binding in mitosis (Fig. 7B,C) in line with a critical role for Cks in recruiting cyclin B1 to APC/C-Cdc20, while wild-type and non-degradable cyclin B1 mutants, with intact Cdk-Cks binding, both bound comparably to APC/C-Cdc20 (Fig. 7B,C)

The N-terminus of cyclin B1, containing the D-box, responds to the spindle checkpoint and starts to be degraded at metaphase, but does not bind Cdk or Cks (Stewart et al., 1994;Clute and Pines, 1999). However, the N-terminus of cyclin B1, over-expressed in mitotic cells, failed to bind detectably to the APC/C (Fig. 7D). APC/C-binding was also not found when cells were released from the mitotic checkpoint and kept in mitosis by proteasome inhibition (Fig. 7E, cycB1-N (1X)-GFP), showing that the interaction between the cyclin B1 D-box region and the phospho-APC/C is of low affinity. Indeed, in extracts of post-checkpoint arrested mitotic cells, we found that it required a six-fold repeat of the cyclin B1 N-terminal region to detect interactions with APC/C-Cdc20 (Fig. 7E). Apparently, whereas upon silencing of the spindle checkpoint the D-box is required for APC/C-Cdc20 to recognize cyclin B1 as a substrate, the D-box region in itself is insufficient to promote stable interactions between cyclin B1 and mitotic APC/C-Cdc20. We therefore propose that Cdk1- and Cks-dependent binding of cyclin B1 to the APC/C-Cdc20 in prometaphase is required to ensure that D-box dependent processing of cyclin B1 is highly efficient after the checkpoint becomes satisfied in metaphase. In other words, we propose that binding to Cks, by Cdk1, makes cyclin B1 a better APC/C substrate in cells.

Fusion proteins of the cyclin B1 N-terminus and a reporter protein, lacking the Cdk1 interaction region, are widely used to detect metaphase APC/C activity in cells. However, we noticed that these reporter proteins are degraded very inefficiently, with more than 50% of fluorescence remaining at anaphase, whereas fluorescent versions of full-length cyclin B1 are almost completely degraded before anaphase. We aimed to validate that Cks is critical for efficient cyclin B1 destruction in metaphase, independent of Cdk1-kinase activity. Therefore, we fused the Cks1 protein to a fluorescent fusion protein of the N-terminus of cyclin B1 and tested its behavior in mitotic cells. The N-terminus of cyclin B1 does not bind to APC/C or Cdc20 (Fig. 8A, input and total APC/C phosphorylation in middle panel, binding in lower panel), but fusion of the N-terminus of cyclin B1 to Cks1 restored robust APC/C-Cdc20 binding in prometaphase, to levels comparable as found for wild-type cyclin B1 (Fig. 8A, lower panel). Further, when we analyzed complex formation between APC/C-Cdc20 in sucrose gradients of prometaphase cell extracts, we found that up to 40% of endogenous cyclin B1 co-migrated with APC/C-Cdc20, whereas the N-terminal cyclin B1 fragment migrated closer to Cdh1, the G1 phase APC/C activator which does not bind to the APC/C in prometaphase (Fig. 8B, top panel). Interestingly, fusing Cks1 to the N-terminal cyclin B1 fragment, shifted this protein in the gradient so it now


A
cycB1 Venus
ΔN-cycB1 ECFP
Fluorescence (%)
Time (min)
M
Securin Cerulean
cycB1 Venus
A
cycB1-R202A Venus
B
cycB1-R202A Venus
ΔN-cycB1 ECFP
cycB1 Venus
WCE (mitotic)
GFP-IP
APC4
Cdc20
Cdk1
C
cycB1 Venus
cycB1-R202A Venus
cycB1-N GFP
cycB1-N-R42A GFP
cycB1-R42A GFP
WCE (Mitotic)
GFP-IP
Cdc20
GFP
D
Mitosis
Asynch.
cycB1-N GFP
cycB1-N-(R42A) GFP
cycB1-IP
GFP-IP
APC3
cycB1
cycB1-N GFP
anti-cycB1 blot
E
ctrl
cycB1-N (1x) GFP
cycB1-N (2x) GFP
cycB1-N (6x) GFP
WCE
GFP-IP
APC3
APC3 Ie
Cdc20
Cdk1
IgG
(mitotic, checkpoint-released, MG132)
F
ctrl
cycB1-N (1x) GFP
cycB1-N (2x) GFP
cycB1-N (6x) GFP
GFP-IP
cycB1-N (6x) GFP
cycB1-N (2x) GFP
cycB1-N (1x) GFP
anti-cycB1 blot

co-migrated with APC/C-Cdc20 complexes and endogenous cyclin B1, whereas Cdh1, of similar molecular weight as the cyclin B1-N-Cks fusion, remained in the fractions lacking APC/C-Cdc20 (Fig. 8B, lower panel).

Finally, fusing Cks1 to the N-terminal cyclin B1 fragment reporter protein resulted in an intracellular localization that was similar to that observed for wild-type cyclin B1 in mitosis (Fig. 8C), with a slight enrichment on spindle poles. This reporter was degraded with high efficiency in metaphase, at kinetics identical to those of full-length wild-type cyclin B1, whereas the N-terminal cyclin B1 fragment alone was degraded too slowly (Fig. 8C,D; Supplemental Fig. 2C). This shows a direct role for Cks in controlling the binding of cyclin B1-Cdk1 to the APC/C, independent of associated Cdk1 activity, to ensure efficient processing of cyclin B1 as an APC/C substrate in metaphase.

◁ **Figure 7. APC/C-Cdc20 binding to cyclin B1 correlates with a substrate retention step, preceding a D-box dependent ubiquitination step.** (A) Cells were co-transfected with vectors coding for cyclin B1-Venus and ΔN-cyclin B1-ECFP (left), Securin-Cerulean and cyclin B1-Venus (middle) or Securin-Cerulean and cyclin B1-R202A-Venus (right). Cells expressing both proteins were followed through mitosis and fluorescence levels were plotted. Metaphase (M) and anaphase initiation (A), judged from DIC images, are shown. Graphs are representative of at least 2 independent experiments. The cells analyzed (middle and right panels) expressed cyclin B1 or Securin at low levels, allowing onset of their destruction at chromosome alignment. With wild-type constructs, sister chromatid separation and cytokinesis were normal. The other fluorescent constructs were expressed to similar levels. At least 20 cells expressing single fusion proteins were analyzed with similar results. (B,C) Wildtype and non-degradable mutants of cyclin B1 bind equally well to APC/C-Cdc20 in mitosis while non-Cdk binding mutants of cylin B1 do not bind. B: cells expressing Cdk-binding mutant cyclin B1-R202A-Venus (lane 1), non-degradable ΔN-cyclin B1-ECFP (lane 2) or control cyclin B1-Venus (lane 3) were synchronized in the spindle checkpoint and collected by mitotic shake-off. Successful expression of the constructs was validated by fluorescence microscopy (unpublished data). C: cells expressing control cyclin B1-Venus (lane 1), Cdk-binding mutant cyclin B1-R202A-Venus (lane 2), the non-Cdk binding N-terminal destruction region of cyclin B1 fused to GFP (lane 3), or non-degradable R42A D-box mutants (lane 4,5) were synchronized in the spindle checkpoint and collected by mitotic shake-off. B,C: fusion-proteins were IP-ed by anti-GFP antibodies, equally recognizing GFP or spectral variants. IPs were blotted and probed with antibodies against the indicated proteins. (D) Cells were treated with Nocodazole and cyclin B1-IPs were performed on extracts of the collected mitotic cells (lane 1) or on extracts from an asynchronous population (lane 2). Cells expressing the first 97 amino-acids of cyclin B1 fused to GFP, either containing an intact D-box (lane 3) or a R42A mutated D-box (lane 4) were collected in mitosis. Anti-GFP antibodies were used to IP the fusion proteins from extracts (lane 3 and 4). IPs were probed for cyclin B1 and APC3. First lane shows binding of APC/C to endogenous cyclin B1 in mitosis as reference. In prometaphase, binding is not detectable to the N-terminal fragments of cyclin B1. (E,F) Mitotic cells were collected by shake-off after Taxol treatment and released from the spindle checkpoint by addition of Aurora B inhibitor ZM447439, but kept in mitosis by the addition of proteasome inhibitor MG132. After 1 hour cells were lysed and extracts used to IP fusion proteins of GFP and the cyclin B1 N-terminus (1-86), (cycB1-N (1X)-GFP) or tandem or six-fold repeats of the same region (cycB1-N (2X)-GFP, cycB1-N (6X)-GFP, respectively). IPs were blotted for APC3, Cdc20 or Cdk1 (as a negative control) (E), or the cyclin B1 fragments (F). 'APC3 le' (long exposure) is an overexposed blot to reveal minor APC/C binding to the single or tandem-repeat cyclin B1 fragment.

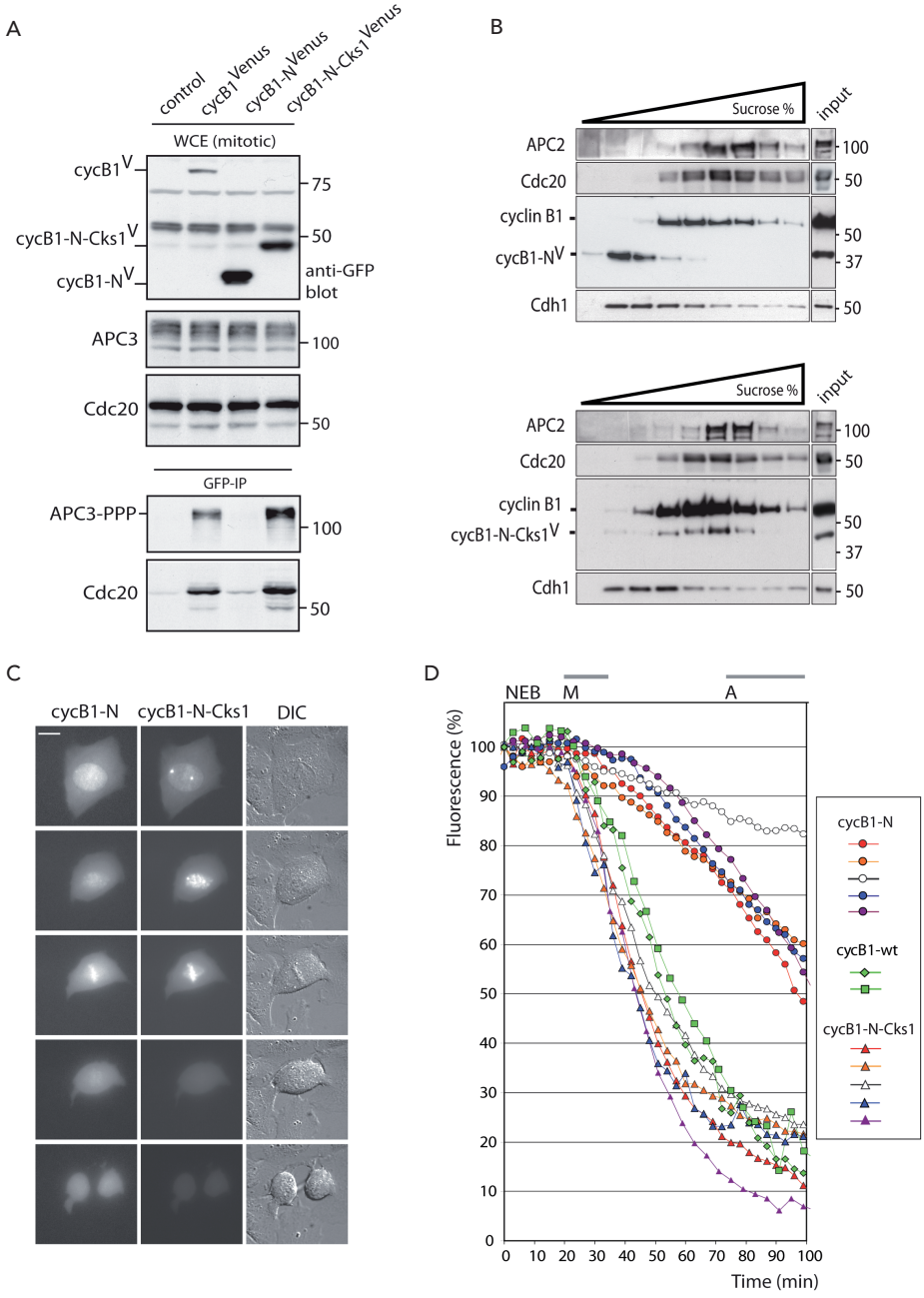


Figure 8. Cks enhances destruction of the N-terminal cyclin B1 fragment. (A) Cells expressing cyclin B1-Venus (lane 2), the first 86 amino-acids of cyclin B1 fused to Venus (cycB1-N-Venus, lane 3) or the same N-terminal cyclin B1 fragment fused to Cks1 and Venus (cycB1-N-Cks1-Venus, lane 4) were synchronized in the spindle checkpoint and mitotic cells collected by mitotic shake-off. GFP-IPs on equal amounts of protein precipitated the Venus-fusion proteins, which were blotted to examine co-precipitation of indicated proteins (lower panel). (B) Spindle checkpoint-arrested ▷

Discussion

Here we studied how cyclin B1 is recruited to the APC/C in mitotic cells. We found that a significant fraction of cyclin B1 is bound to the phosphorylated APC/C in an APC3-dependent, but D-box independent manner, even when the spindle checkpoint stabilizes cyclin B1.

The APC/C from Xenopus mitotic extracts binds an N-terminal, D-box containing fragment of fission yeast cyclin B (Yamano et al., 2004). In contrast, we did not detect binding of an ectopically expressed N-terminal region of cyclin B1 and the phosphorylated APC/C in human mitotic cell extracts, arrested by proteasomal inhibition, unless we over-expressed a six-fold repeat of the D-box containing cyclin B1 N-terminus. Although specific for an intact D-box, published in vitro interactions of the fission yeast cyclin B D-box fragment and Xenopus APC/C were also reported to be of low affinity, requiring high concentrations of the D-box region (Yamano et al., 2004; Eytan et al., 2006).

Figures 2, 4, 7 and 8 show that cyclin B1 depends on Cdk1-Cks for recruitment to the APC/C, not just as a kinase but also as an APC/C substrate. Cks-mediated 'pre-recruitment' of cyclin B1 becomes effective after the spindle checkpoint is satisfied in metaphase and supports efficient cyclin B1 destruction. The pool of cyclin B1-Cdk1-Cks that was not pre-bound to the mitotic APC/C in prometaphase may flux more efficiently onto the APC/C in metaphase. Whereas we show that cyclin B1-Cdk1-Cks plays a role in activating overall APC/C-Cdc20 activity, too, we thus demonstrate an additional, APC/C-docking function of Cks, facilitating cyclin B1 destruction. In Xenopus extracts, Cks was reported to predominantly direct total APC/C activity (Patra and Dunphy, 1998). Apart from evolutionary differences, possibly higher levels of APC/C activity in extracts arrested by non-degradable cyclin B1, differences in processivity of recombinant cyclin B1 as compared to endogenous cyclin B1, or reduced time-resolution in comparison to live-cell microscopy studies may explain why an APC/C-docking role for Cks was not found in Xenopus extracts.

▷ mitotic cells expressing cycB1-N-Venus or cycB1-N-Cks-Venus were collected and lysates were subjected to sucrose gradient centrifugation. Fractions were blotted and probed with antibodies against indicated proteins. (C) U2OS cells were co-transfected with vectors encoding cycB1-N-Cerulean (cycB1-N) and cycB1-N-Cks-Venus (cycB1-N-Cks1) and followed as they progressed through mitosis. Scale bar is 10μM. (D) Venus- and Cerulean-tagged versions of CycB1-N or CycB1-N-Cks1 were co-transfected in U2OS cells. Cells either expressing CycB1-N or CycB1-N-Cks1 were followed during mitosis and fluorescence levels were measured at NEB and plotted over time. For comparison, cells were also simultaneously transfected with three differently colored constructs, cycB1-N-Venus, cycB1-N-Cks1-Cerulean and a full length, red fluorescent cyclin B1 construct, cycB1-Cherry. Fluorescence levels of two triple colored cells passing mitosis and performing a normal anaphase were included in the graph as a reference. Results of two independent experiments are shown here. M, A indicate onset of metaphase or anaphase, respectively, in different cells. Comparable differences in destruction of the cyclin B1 reporters were found in more than 30 cells in four independent experiments. We found no significant effect of the different fluorescent tags on the destruction rates of the cyclin B1 fragments (Supplemental Fig 2C).

Proteasome-dependent protein destruction depends on a ubiquitin chain of significant length, so productive poly-ubiquitination either requires sequential rounds of encounters between ubiquitin ligase and substrate, or the ability of the ubiquitination enzyme to retain its substrate while ubiquitin chains are formed (Rape et al., 2006). Cks-dependent retention of cyclin B1 to the APC/C may thus enhance processivity of cyclin B1 ubiquitination in human cells, facilitating switch-like turn-over in metaphase. This is physiologically relevant as it can protect against a cytokinesis defect and mitotic delay (Wolf et al., 2006; reviewed in Sullivan and Morgan, 2007). Recently, we observed that slow, APC/C-Cdc20-dependent loss of cyclin B1, which permits cells to slip out of a spindle-drug induced mitotic arrest (Brito and Rieder, 2004;Huang and Mitchison, 2009), may also depend on the substrate capturing mechanism we describe here (unpublished data). This provides additional evidence that cyclin B1 binds to spindle checkpoint-inhibited APC/C-Cdc20 as a substrate. It also suggests further implications of our findings, since mitotic slippage could contribute to survival of cancer cells after treatment with anti-mitotic cancer drugs (Huang and Mitchison, 2009). In this respect it is also interesting that Cks1 and Cks2 are often up-regulated in cancer ('t Veer et al., 2002;Inui et al., 2003;Kitajima et al., 2004;Shapira et al., 2005;Masuda et al., 2003;Keller et al., 2007).

Apparently, an APC/C targeting mechanism by Cks is shared between cyclin A and cyclin B1. This sheds light on the different timings of their destruction. We found that spindle-checkpoint independent destruction of cyclin A is preceded by strong binding of Cdc20 to the N-terminal destruction motif of A-type cyclins in G2 phase. Such binding is probably direct (Ohtoshi et al., 2000) and not found for cyclin B1 (Wolthuis et al., 2008; this study). This indicates that the N-terminus of cyclin A is unique in that it effectively competes with spindle checkpoint proteins for Cdc20 binding. Interestingly, Mad2 co-immunoprecipitates with the cyclin B1-APC/C-Cdc20 complexes formed in mitosis, whereas cyclin A-Cdc20 complexes bind poorly to Mad2 (Wolthuis et al., 2008; this study). In conclusion, both mitotic cyclins are recruited to the prometaphase APC/C-Cdc20 in a manner dependent on Cks, yet only cyclin A is immediately processed and critically requires Cks for timely onset of destruction. Cdc20 bound to the N-terminus of cyclin A evades checkpoint control (van Zon and Wolthuis, 2010). Cyclin B1 is also recruited to phosphorylated APC/C-Cdc20 in a Cks-dependent and spindle checkpoint-independent manner, but D-box dependent processing of the APC/C-bound cyclin B1 awaits inactivation of the checkpoint.

As the spindle checkpoint does not prevent formation of stable complexes between cyclin B1, the phosphorylated APC/C and Cdc20, our results imply that release of the checkpoint triggers APC/C-Cdc20 to bind or recognize the cyclin B1 D–box as a step secondary to cyclin B1 recruitment. APC/C complexes isolated from checkpoint-arrested cells indeed interacted better to recombinant N-terminal fragments of fission yeast cyclin B1 upon checkpoint-silencing (Herzog et al., 2009). In such a successive substrate binding mechanism, D-box binding to APC/C-Cdc20 may control correct orientation of the substrate towards the APC/C catalytic site, as initially suggested

by Burton and Solomon (Burton and Solomon, 2001), or direct a further, Cdc20-dependent APC/C activation (Burton et al., 2005; Kimata et al., 2008; Matyskiela and Morgan, 2009). However, exactly how recognition of the cyclin B1 D-box, as a result of inactivation of the mitotic spindle checkpoint, stimulates cyclin B1 ubiquitination is still unknown.

Materials and Methods

Plasmids. Vectors expressing cyclin B1-Venus or Cerulean, ΔN-cyclin B1-ECFP and cyclin B1-R202A-Venus were kindly provided by Jon Pines and Takahiro Matsumoto (The Wellcome Trust/Cancer Research UK Gurdon Institute). Vectors expressing the N-terminal cyclin B1 fragments used in Figure 7D were a kind gift of Michael Brandeis (The Hebrew University of Jerusalem, Jerusalem, Israel). The other N-terminal cyclin B1 constructs were made by replacing full-length cyclin B1 from the cyclin B1-Venus or Cerulean vectors with a PCR fragment coding for the first 86 amino-acids of cyclin B1, to generate cycB1-N-Venus or the Cerulean version. Two- and six-fold repeats of this region (Fig. 7) were generated by PCR. Insertion of another PCR fragment coding for full length Cks1 (from Cks1-YFP, described in (Wolthuis et al., 2008)) resulted in cycB1-N-Cks1-Venus or a Cerulean version. The generation of vectors expressing WT-Securin-Venus, Cdk1*-EYFP and Cdk1*-1N-EYFP are described elsewhere (Wolthuis et al., 2008). GFP-INCENP was a kind gift of Gerben Vader (The Whitehead Institute for Biomedical Research, Cambridge, MA) and is described in (Vader et al., 2006). To generate the cyclin B1-Cherry construct, a fragment coding cyclin B1, from cyclin B1-Venus, was ligated in a Cherry expression-vector (Lennert Janssen, NKI-AVL, Amsterdam, The Netherlands). All generated constructs were sequence verified.

Cell culture, Transfection, Selection and Synchronization. The human osteosarcoma cell line U2OS was cultured in D-MEM in the presence of 8% FCS with 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were transfected with plasmids using a standard calcium phosphate transfection protocol. For the transfections of siRNA pools we used lipofectamine 2000 according to manufacturer's instructions. Cells were seeded in 100 mm dishes and (co-)transfected with 10 or 20 μg of the indicated pSuper-shRNAi constructs and/or 1 μg of indicated expression constructs and co-transfected with 1 μg pBabePuro for selection of transfected cells with puromycin, which was used in a 2 μg/ml end concentration during 24 hrs. Mitotic cells were collected by a gentle mitotic shake off, either arrested by APC3-RNAi, APC2-RNAi or arrested with an active spindle checkpoint by adding Nocodazole to the culture medium at a final concentration of 250 ng/ml for 15 hrs or 12 hours after Thymidine arrest (the latter also for Supplemental Fig. 3A, but without any drugs, leaving the mitotic cells unperturbed). To collect a G2 population, cells were first blocked in the G1/S transition by Thymidine in a final concentration of 2.5 mM for 24 hrs. Cells were released from the G1/S arrest in the presence of Nocodazole. After 12 hrs mitotic cells were removed after which plates were thoroughly washed and remaining G2 cells were

collected. To obtain spindle checkpoint-inactivated mitotic cells, cells were released from a Nocodazole arrest in the presence of the proteasome inhibitor MG132 (5 μM). Mitotic cells that had entered mitosis in the presence of MG132, added 10 hours after release from Thymidine, were collected 15 hours after release. Roscovitine was used to force the mitotic cells into G1 at a final concentration of 50 μM for 45 minutes. In the experiment shown in Fig. 7E, cells collected by mitotic shake-off after Taxol treatment, were released from spindle checkpoint arrest by addition of the Aurora A inhibitor ZM447439 (1μM) and kept in mitosis by simultaneous addition of MG132. In this case, complete checkpoint release was confirmed by monitoring formation of a 4N G1 population in a fraction of cells to which no MG132 was added. Flow cytometry was used to confirm synchrony: cells indicated as G2 were usually more than 90% 4N as detected by PI staining, and less that 10% MPM2 positive, whereas mitotic cells collected by careful mitotic shake-off were 90-95% 4N, and stained >90% positive for the mitotic marker MPM2. When Nocodazole arrested cells were collected by mitotic shake-off and the drug was washed out, >90% of the cells divided within two hours as detected by a near complete shift to 2N G1 cells. For flow cytometry, cells were fixed in ice-cold 70% ethanol and stained with mouse anti-MPM2 antibody (UBI) and CY-5 coupled anti-mouse (DAKO). Cell cycle distribution and MPM2 positivity of transfected cells was determined as described (van Vugt et al., 2004).

RNAi. pSuper vectors were used for expression of shRNAi targeting human APC3 mRNA. Hybridized oligos containing specific 19-mer target sequences were ligated into pSuper (sequence TAGCCGAGAGGTAACTCCA). For pSuper APC2 see Supplementary Figure 5C. The pSuper vectors targeting mRNA of Cdk1 or BubR1 were previously described (Lindqvist et al., 2007;Lens et al., 2003). Annealed siRNA pools to target mRNA of APC3, APC2, Cdc20, Cks1, Cks2 and Plk1 were purchased from Dharmacon as ON-TARGET-plus SMART pools, order numbers L-003229-00 for H.s. APC3/Cdc27, L-003200-00 for H.s. APC2, L-003225-00 for H.s. Cdc20, L-004586-00 for H.s. Cks1B, L-007678-00 for H.s. Cks2 and L-003290-00 for H.s. Plk1.

Immuno-precipitations and Western blots. Cells were lysed in ELB+ (150 mM NaCl, 50 mM HEPES, pH 7.5, 5 mM EDTA, 0.3% NP-40, 10 mM β-glycerophosphate, 6% Glycerol, 5 mM NaF, 1 mM Na2VO3, and protease inhibitor cocktail (Roche)). Lysates were cleared by centrifugation at 13,000 g for 8 minutes at 4°C. Protein levels were measured using standard Bradford analysis. For each IP we pre-coupled 2 μg antibodies to 20 μl of Protein G Sepharose (Amersham Biosciences) overnight. Washed pre-coupled beads and cleared extracts were incubated 3-12 hours at 4°C. Immuno-complexes were washed four times with ELB and collected in 50 μl protein sample buffer or directly used in ubiquitination assays. 25 μl was separated on polyacrylamide gels and blotted to nitrocellulose. For immuno-precipitations the following antibodies were used: goat anti-Cdc16/APC6 (Santa Cruz), goat anti-APC4 (Santa Cruz, C18), rabbit anti-Cdc20 (Santa Cruz, H-175), rabbit anti-cyclin A (Santa Cruz, H-432), rabbit anti-cyclin B1 (Santa Cruz, H-433), rabbit anti-Securin (Zymed), goat anti-Cdk4 (Santa Cruz, C-2) and rabbit anti-GFP (Neefjes lab, NKI, Amsterdam).

For detecting proteins the following antibodies were used: rabbit anti-APC2 (kind gift from Dr. Hongtao Yu, University of Texas Southwestern Medical Center, Dallas, TX), mouse anti-APC3/Cdc27 (Transduction Labs), goat anti-APC4 (Santa Cruz, C18), rabbit anti-GFP [26], mouse anti-HA (Sigma, HA-7), mouse anti-Mad2 (MBL), mouse anti-cyclin A (Neomarkers), mouse anti-cyclin B1 (GNS1), mouse anti-Securin (Abcam), rabbit anti-Plk1 (Zymed), mouse anti-Cdc20 (Santa Cruz, E7), mouse anti-Cdh1 (Neomarkers), mouse anti-Cdk1 (Transduction Labs) and rabbit anti-Cdk2 (Santa Cruz, M2). Secondary, PO conjugated antibodies were from DAKO. Quantitation of Western blots was performed by comparing the different exposure times of autoradiograms to blots, avoiding saturation of the film. Estimation of the amount of cyclin B1 associated with APC/C was on the basis of the 'fold enrichment' of Cdk1 signal in cyclin B1 IPs (in relation to the Cdk1 signals in whole cell extracts, run on the same blots), as compared to the fold enrichment of APC/C subunits or Cdc20 in the same cyclin B1 IPs. Top regions of the blots were probed for APC/C subunits, and lower regions for Cdk1. Typically, in our (non-saturating) cyclin B1 IP conditions, we collected no detectable cyclin A or Securin, and approximately 5% of the cellular pool of APC2 or APC3 and 10-20% of the cellular pool of Cdk1 (e.g. see Fig. 5D lower panel; IPs are loaded next to 10% of the whole cell extracts on the same blots). Calculations of the concentration of mitotic cyclin B1 in U2OS cells are in the order of magnitude of 10 nM or less (Arooz et al., 2000), whereas Cdk1 and APC3 are in the range of 100 nM (Arooz et al., 2000;Kulukian et al., 2008). This suggests that at least 25-50% of endogenous cyclin B1 is in complex with mitotic APC/C. This fits well with our findings in an alternative approach: in sucrose gradients shown in Fig. 8, we found approximately 40% of endogenous cyclin B1 or Cks1-tagged cyclin B1 N-terminal fragment co-migrated with the peak of APC/C-Cdc20.

Sucrose gradient centrifugation. Spindle checkpoint-arrested mitotic cells expressing cycB1-N-Venus or cycB1-N-Cks-Venus were collected and lysates were subjected to sucrose gradient centrifugation. Gradients were made using 15-40% sucrose solutions. Mitotic lysates (collected from four 9 cm dishes of Nocodazole treated, transfected cells) were loaded on 10 ml of gradient and centrifuged in a swinging bucket SW 41 Ti type rotor at 4˚C for 20 hours at 40.000 rpm. 1 ml fractions were collected. 20 µl of the indicated fractions were blotted and probed with antibodies to detect co-migration of expressed cyclin B1 fragments with endogenous cyclin B1, APC/C, Cdc20 or Cdh1.

Ubiquitination assay. As a source of APC/C, cyclin B1 IPs were performed as described in the 'Immuno-precipitations and Western blot' section, using equal amounts of protein from cells synchronized in G2, 8h after Thymidine release (G2) or from mitotic cells collected by mitotic shake off (after Thymidine release plus Nocodazole treatment). The latter cells were released from Nocodazole but kept in mitosis by addition of the proteasome inhibitor MG132 (NR, MG132). Control GFP-IPs were performed on Nocodazole-arrested mitotic cells (N). IPs were washed twice with ELB+ and twice with Protein buffer (20 mM Hepes pH7.8, 2.5% glycerol, 6 mM MgCl2,

30 mM KCl and 2mM β-ME). Ubiquitination reactions were performed in Protein buffer containing 1 µg HA-tagged Ubiquitin, 0.1 µg His-tagged E1, 0.5 µg His-tagged E2 (UbcH10) and 5 mM ATP. Human HA-Ubiquitin and human His-E2 (UbcH10) were from Boston Biochemicals. Human His-E1 and E2-25K were kind gifts of Titia Sixma (NKI-AVL, Amsterdam, The Netherlands). Reactions were started by addition of the IPs, incubated for 30 minutes at 30°C. Samples were separated on 10% SDS-page gel, blotted to nitrocellulose and probed with mouse anti-HA antibodies. For the assays in Supplemental Fig. 3, cyclin B1 was precipitated from asynchronous cells and 'NR,MG132' cells (E,F) or G2, M and G1 cells (G). Purified Securin-His was added to the E1, E2, ubiquitin and ATP mix, as an in vitro APC/C substrate of the ubiquitination assay. The Securin-His vector for protein expression in bacteria was a kind gift of Jon Pines (The Wellcome Trust/Cancer Research UK Gurdon Institute) (Nilsson et al., 2008). Recombinant protein purification procedures will be published elsewhere. The in vitro reactions were blotted and probed with anti-Securin antibodies. Further controls are shown in Supplemental Fig. 3.

Time-lapse Fluorescence Microscopy. Cells plated on 35 mm glass-bottom culture dishes (Willco-dish, Amsterdam, The Netherlands) were transfected the following day with 0.1 µg of the indicated expression constructs, except for Securin-Venus (0.015 µg) and N-terminal cyclin B1 constructs (0.025 µg). For shRNAi, 1 µg of pSuper-Cdk1 was co-expressed with indicated constructs (together with 1 µg pSuper-BubR1 for Fig. 1E, right panel). Dishes were transferred to a heated culture chamber (37°C, 5% CO2) on a Zeiss Axiovert 200M microscope, equipped with a 0.55 numeric aperture condenser and a 40X Plan-Neo DIC objective (N.A. 1.3) using a Photometrics Coolsnap HQ CCD camera (Scientific, Tucson, AZ) with a GFP/DsRed or CFP/YFP dual band pass filter set (Chroma Technology Corp. Rockingham, VT) to select specific fluorescence. Images were taken after 100 msec exposure time, at 2x binning for the cyclin B1 and Securin experiments, and quantitated and processed using MetaMorph software (Universal Imaging, Downington, PA) and Microsoft Excel.

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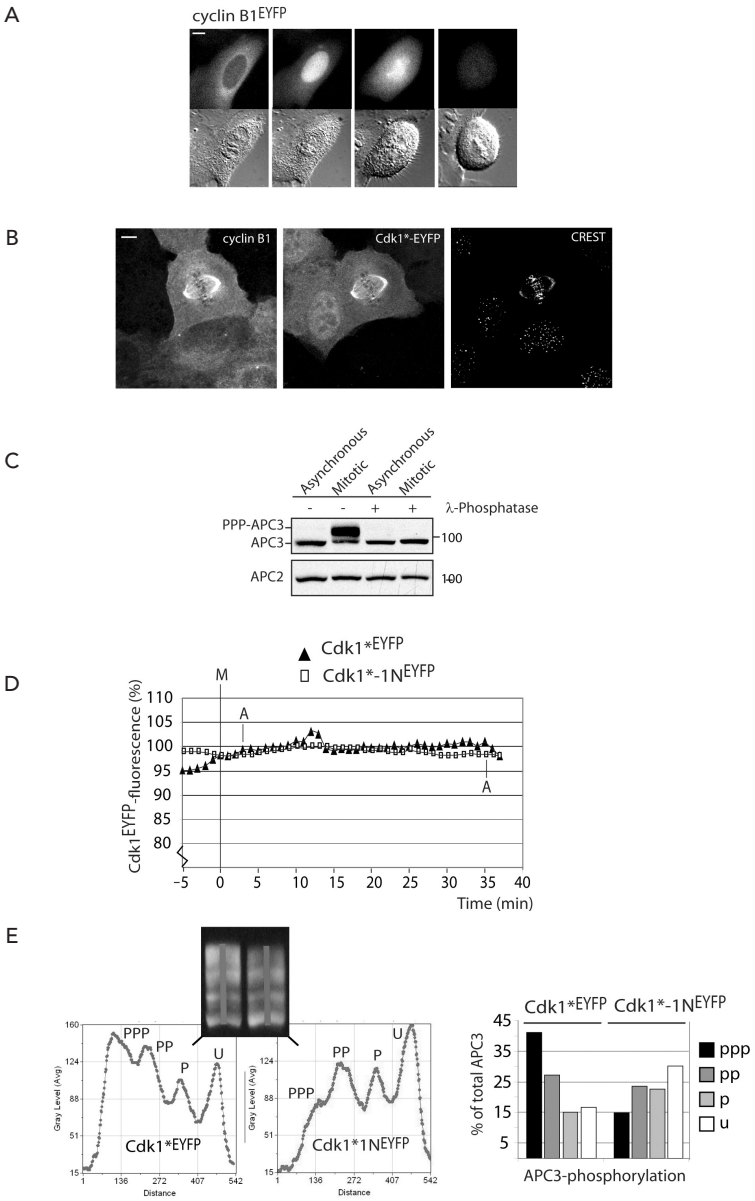
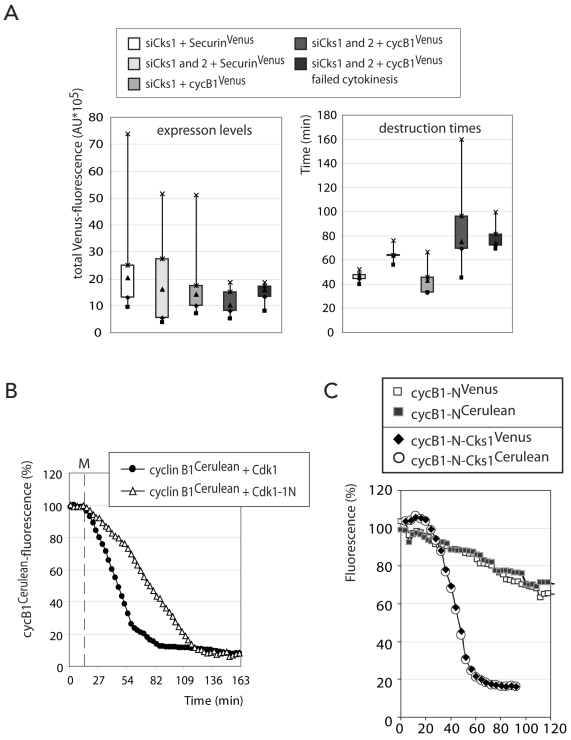
Supplementary materials

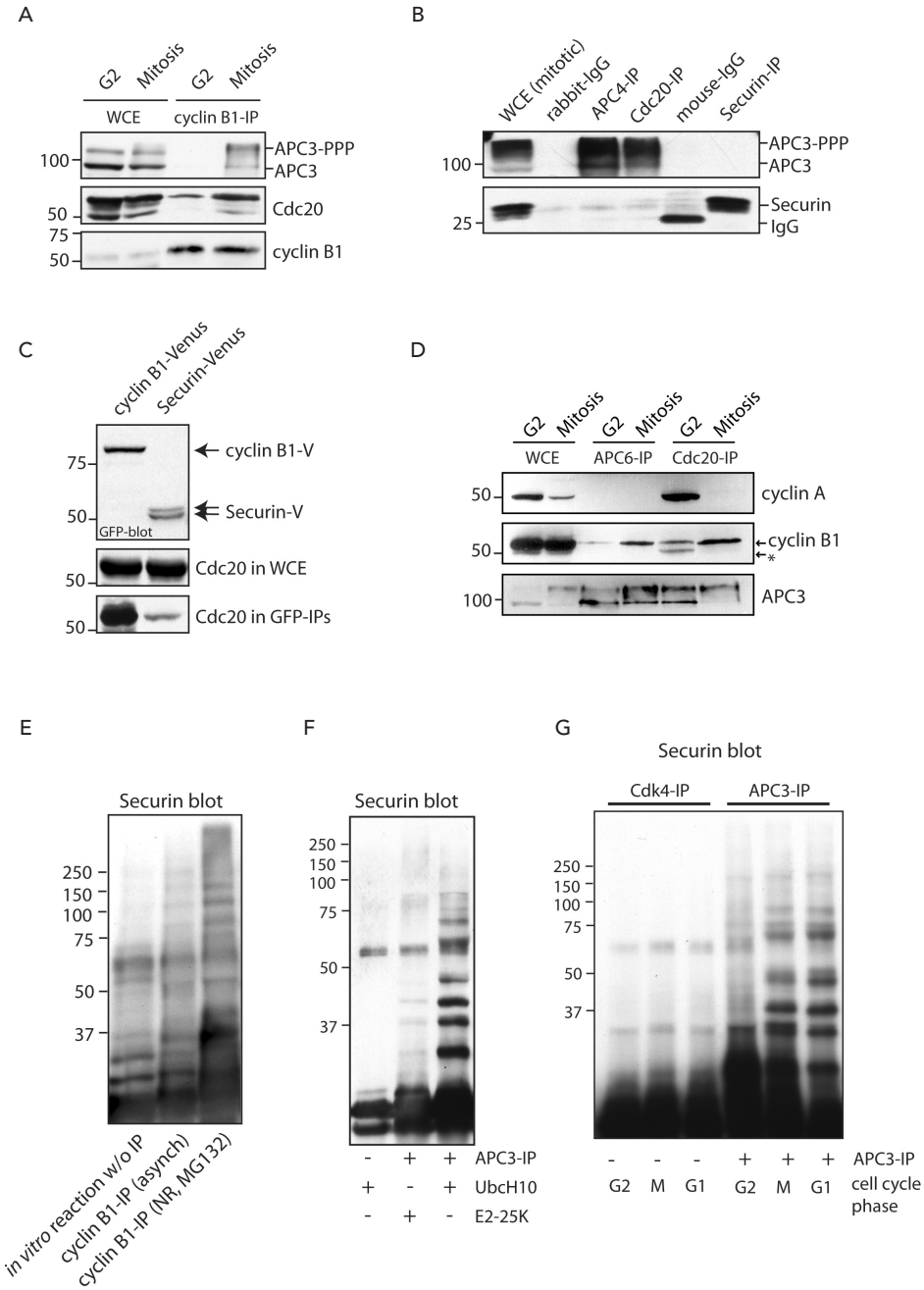


Supplemental Figure 1. (A) Live-cell distribution of cyclin B1-Venus in mitosis. U2OS cells were transfected with cyclin B1-Venus and followed through mitosis. Fluorescence (top) and DIC images (bottom) of these cells show cyclin B1 translocating from cytoplasm to nucleus in prophase, staining the spindle poles and chromosomes in prometaphase (see Clute and Pines, 1999). Scale bar is 5 μm. (B) **Kinetochore localization of fluorescent Cdk1 revealed by complementation of endogenous Cdk1.** Rescue of Cdk1 depleted cells by Cdk1*-EYFP reveals co-localisation ▷



Supplemental Figure 2. (A) Total destruction times of cyclin B1-Venus and Securin-Venus in cells treated with the indicated siRNA pools were compared. Destruction was measured as the point when at least 1% of fluorescence declined and continued to decline until decline was less than 1% per 2 minutes. Left box plots show fluorescence levels at the start of destruction. The square is the minimal value, the diamond is the 1st quartile, the triangle is the median value, the asterisk is the 3rd quartile and the X is the maximum value. (B) Cells were co-transfected with cyclin B1-Cerulean and either Cdk1*-EYFP or Cdk1*-1N-EYFP and fluorescence levels were plotted over time, see Fig. 4. Shown is destruction of cyclin B1, bound to Cdk1 (black circles), or bound to Cdk1-1N (white triangles). M indicates metaphase as judged from DIC images. C) Cells were co-transfected with two spectral variants of cycB1-N or cycB1-N-Cks1 and fluorescence levels were measured and plotted over time. A graph is shown representative of 17 cells in 4 independent experiments.

▷ with endogenous cyclin B1 at the mitotic spindle and kinetochores. Confocal images of fixed cells. Left panel: mouse anti-cyclin B1/Alexa-568-anti-mouse antibodies; middle panel: ectopic Cdk1*-EYFP; right panel: CREST stain of centromeres/Alexa 647-anti-human antibodies. Cdk1 also co-localizes with cyclin B1 on chromosome arms, but this signal is lost upon the used fixation protocol. For methods see (Lindqvist et al., 2007). Scale bar is 5 µm. (C) **Phosphorylation dependent gel shift of APC3**. Mitotic and asynchronous U2OS cells were lysed in ELB without phosphatase inhibitors. Phosphatase treatment was performed using lambda phosphatase, according to the specifications of the supplier (NEB). The samples were blotted and probed with antibodies against the indicated proteins. (D) **Stability of fluorescent Cdk1 constructs in mitosis.** Cdk1 depleted cells complemented with Cdk1*-EYFP or Cdk1*-1N-EYFP were followed during mitosis and fluorescence levels were plotted over time. M indicates Metaphase and A indicates Anaphase as judged from DIC images. The graph is a representative example of such cells, also showing that cells without the Cdk1-Cks connection delay in metaphase. (E) **Quantification of Cks-dependent APC3 phosphorylation.** The intensities of the different forms of APC3, separated on gel, from mitotic Cdk1-depleted cells, complemented with either Cdk1*-EYFP, or Cdk1*-1N-EYFP, were quantified using Metamorph software using a 30 pixel-wide line scan, shown in green for Cdk1*-EYFP and in red for Cdk1*-1N-EYFP. The right bar graph shows the quantification of the different phosphorylated forms of APC3 (ppp, pp, and p) and the unphosphorylated form of APC3 (u) in percentages of total APC3. The average with standard deviation measured from 4 independent experiments, using the same approach, is shown in Fig. 1G.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G2
Mitosis
G2
Mitosis
WCE
cyclin B1-IP
100
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APC3
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Cdc20
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B
WCE (mitotic)
rabbit-IgG
APC4-IP
Cdc20-IP
mouse-IgG
Securin-IP
100
APC3-PPP
APC3
25
Securin
IgG
C
cyclin B1-Venus
Securin-Venus
75
cyclin B1-V
50
Securin-V
GFP-blot
50
Cdc20 in WCE
50
Cdc20 in GFP-IPs
D
G2
Mitosis
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Mitosis
WCE
APC6-IP
Cdc20-IP
50
cyclin A
50
cyclin B1
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100
APC3
E
Securin blot
250
150
100
75
50
37
in vitro reaction w/o IP
cyclin B1-IP (asynch)
cyclin B1-IP (NR, MG132)
F
Securin blot
250
150
100
75
50
37
- + + APC3-IP
+ - + UbcH10
- + - E2-25K
G
Securin blot
Cdk4-IP
APC3-IP
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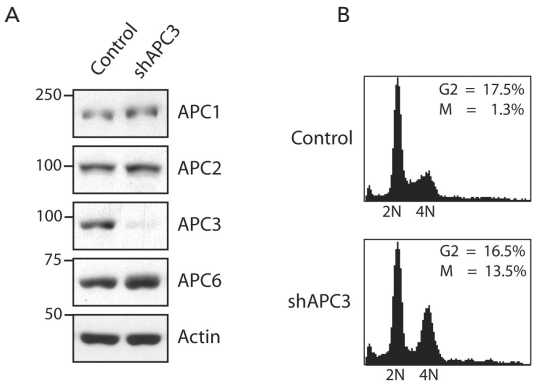
37
- - - + + + APC3-IP
G2 M G1 G2 M G1 cell cycle phase

◁ **Supplemental Figure 3.** (A) Unperturbed mitotic cells collected after thymidine release and G2 cells were subjected to immunoprecipitation using cyclin B1 antibodies. IPs and extracts were blotted and probed with indicated antibodies. (B) IPs with anti-APC4, anti-Cdc20, anti-Securin and control mouse and rabbit antibodies on extracts of mitotic cells arrested in the spindle checkpoint. The whole cell extract (WCE) and IPs were blotted and analysed for co-precipitation of APC3 and Securin. (C) IPs with anti-GFP antibodies were performed on extracts of mitotic U2OS cells arrested in the spindle checkpoint, expressing cyclin B1-Venus or Securin-Venus. The IPs were Western blotted and examined for co-precipitated Cdc20. We performed immunoprecipations with the same antibody on extracts expressing comparable amounts of the two Venus-fusion proteins and equal amounts of Cdc20. (D) IPs with anti-APC6 or anti-Cdc20 antibodies on extracts of a G2-phase population and a Nocodazole-arrested mitotic population, were analyzed for (co-)precipitation of protein by Western blots, probed with antibodies against indicated proteins. The G2 population refers to adherent cells remaining after a mitotic shake-off of cells released from a thymidine block, in the presence of Nocodazole, and collected 15 hrs after thymidine release. These typically contain less than 5 % mitotic cells as determined by MPM2 staining on FACS. In the experiment shown here, a small contamination with remaining mitotic cells (5-10%), detectable by partial APC3 shift, may have caused the detection of cyclin B1-APC/C complexes in this sample in the 'G2' sample. (E,F,G) **APC/C Activity in cyclin B1 and APC/C IPs**. Cyclin B1 IPs or APC/C IPs performed on extracts from cells of the indicated cell cycle phases, with mitotic cells released from the checkpoint arrest but kept in mitosis by proteasome inhibitor MG132 (NR, MG132), were used in in vitro ubiquitination assays. The APC/C substrate Securin was purified and used as substrate in the reactions. Purified E1 enzyme, the APC/C specific E2 enzyme UbcH10, HA-ubiquitin and ATP were added to start the reactions. E) Ubiquitination of Securin by APC/C captured in cyclin B1 IPs. High molecular weight Securin products were only apparent in cyclin B1 IPs from mitotic cells that bind APC/C. F) Ubiquitination of Securin depends on the APC/C-relevant E2. Purified E1 enzyme, the APC/C specific E2 enzyme UbcH10, control E2 enzyme E2-25K, HA-ubiquitin and ATP were added to start the reactions. G) Ubiquitination of Securin depends on the APC/C. Purified E1 enzyme, the APC/C specific E2 enzyme UbcH10, HA-ubiquitin and ATP were added either to Cdk4 IPs or APC3 IPs to start the reactions. Securin is only ubiquitinated by APC/C captured form post-checkpoint mitotic cells or G1 cells. All blots were probed with anti-Securin antibody.


A
Control
shAPC3
250
APC1
100
APC2
100
APC3
75
APC6
50
Actin
B
Control
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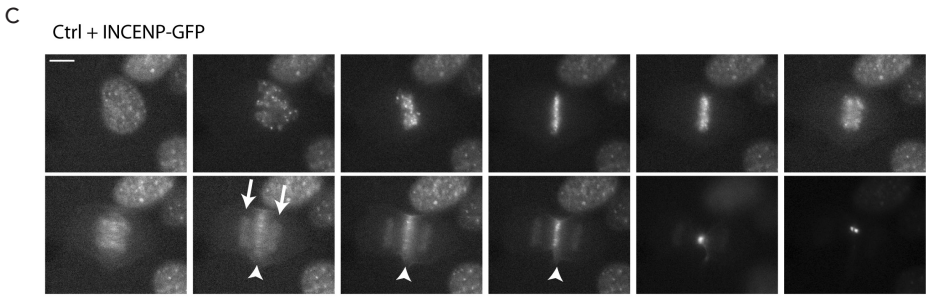
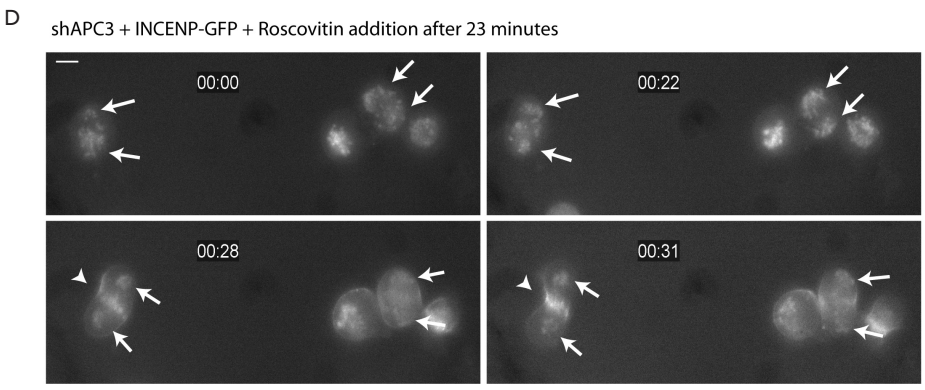
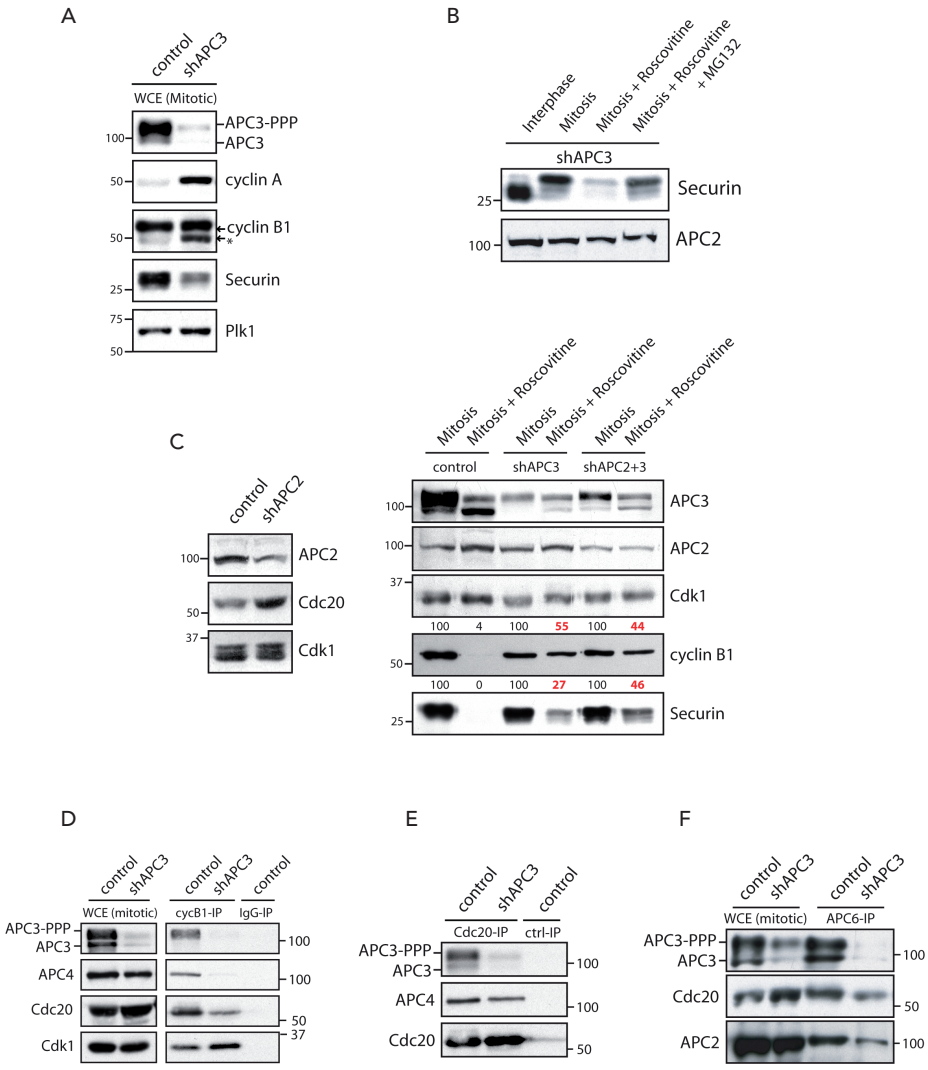
M = 1.3%
2N 4N
shAPC3
G2 = 16.5%
M = 13.5%
2N 4N


C
Ctrl + INCENP-GFP


D
shAPC3 + INCENP-GFP + Roscovitin addition after 23 minutes
00:00
00:22
00:28
00:31

◁ **Supplemental Figure 4. (A) Specific depletion of the APC/C subunit APC3 by RNAi.** Mitotic U2OS extracts were prepared 96 hrs after transfection of pSuper-vector driving shRNAi targeting the APC3 subunit of the APC/C (shAPC3) or control vector (shControl). The cells were cotransfected with pBabePuro to allow selection of transfected cells with puromycin. Extracts of selected cells were blotted and probed with antibodies against the indicated proteins. (B) **APC3 depletion arrests cells in mitosis.** U2OS cells were treated in as in A) and fixed with 70% cold ethanol. After fixation, DNA was stained with Propidium Iodide. The cells were also stained with the mitosis specific MPM2 antibody to determine the G2- and mitotic percentage in the 4N population. FACS analysis of transfected cells (detected by cotransfection of Spectrin-GFP, described in (van Vugt et al., 2004) shows accumulation of 4N cells after APC3 depletion, which was completely attributable to increased mitotic index, revealed by the percentages of MPM2 positive 4N cells (see text in the upper right corner of the plots, G2= MPM2-negative 4N cells, M= MPM2-positive 4N cells). (C) **Cytokinesis followed by GFP-INCENP translocation.** Cells were transfected with 2 µg pSuper and 0.05 µg GFP-INCENP (kindly provided by Gerben Vader, see Vader et al., 2006). The arrows show sister chromatids moving to opposite poles in anaphase. The passenger complex translocates from the chromatin to the spindle-midzone as seen by the appearance of fluorescence signal in between the two genomes (arrow head points to the spindle-midzone). (D) **Mitotic arrest by APC3 depletion and induction of mitotic exit by Cdk inhibition.** Normal cytokinesis initiation in APC3-depleted cells after Cdk inhibition. Cells were transfected with 2 µg pS-APC3 and 0.05 µg GFP-INCENP. Aberrant anaphases were observed, where sister chromatids started to move from the metaphase plate, accumulating at the spindle poles after several hours, but cells remained arrested in mitosis. The arrows show GFP-INCENP on the chromatin at the poles, no midzone fluorescence was detected. When cells were forced to exit mitosis after 23 minutes, by addition of the Cdk-inhibitor Roscovitine, GFP-INCENP jumped to the midzone and cytokinesis initiated. Scale bars are 10 µm.


A
control
shAPC3
WCE (Mitotic)
APC3-PPP
APC3
cyclin A
cyclin B1
Securin
Plk1
B
Interphase
Mitosis
Mitosis + Roscovitine
Mitosis + Roscovitine + MG132
shAPC3
Securin
APC2
C
control
shAPC2
APC2
Cdc20
Cdk1
Mitosis
Mitosis + Roscovitine
control
shAPC3
shAPC2+3
APC3
APC2
Cdk1
cyclin B1
Securin
D
control
shAPC3
WCE (mitotic)
cycB1-IP
IgG-IP
APC3-PPP
APC3
APC4
Cdc20
Cdk1
E
Cdc20-IP
ctrl-IP
APC3-PPP
APC3
APC4
Cdc20
F
WCE (mitotic)
APC6-IP
APC3-PPP
APC3
Cdc20
APC2

◁ **Supplemental Figure 5. (A) APC3 RNAi cells stabilize cyclin B1.** Cells arrested in mitosis with Nocodazole (lane 1) or due to APC3 depletion (lane 2) were collected by mitotic shake-off. Equal protein amounts from these cells were Western blotted and probed with antibodies against the indicated proteins. The cyclin A blot (second panel from top) was reprobed for cyclin B1 (third panel), revealing equal amounts of cyclin B1 in both mitotic populations; the asterisk next to the third panel reveals residual cyclin A signal from first probe. (B) **APC3 RNAi cells partially stabilize Securin.** Proteasome-dependent Securin re-stabilization after APC3 depletion. Securin was re-stabilized by treatment with the proteasome inhibitor MG132, showing that the APC3-independent Securin destruction is still dependent on the ubiquitin-proteasome system. (C) **APC3 depletion stabilizes cyclin B1 better than Securin.** Depletion of APC2 or APC3 by shRNA-vectors. The pSuper-APC2 reduced APC2 to 30-50% of normal levels (targeting sequence: GTCCACACTATGTTGCGCG), stabilizing the APC/C substrate Cdc20 in asynchronous cells (left panel). When we tried to reduce the levels of APC2 and APC3 simultaneously, transfected cells arrested in mitosis with less-than-maximally reduced APC3 abundance as a result of the simultaneous targeting of APC/C activity by APC2 shRNAi (right top panel). However, Securin and cyclin B1 became equally stable, in contrast to depletion of APC3 alone (see quantified levels in percentages above the cyclin B1 and Securin blot, the mitotic sample was set at 100% and next to it the remaining levels after Roscovitine treatment). This indicates a role for the APC/C in Securin destabilization after APC3 depletion and shows cyclin B1 destruction critically depends on APC3. Multiple exposures of the blots were quantified using MetaMorph software and data were processed with Microsoft Excel and corrected for loading using the Cdk1 blots. (D) **APC3 is required for APC/C-Cdc20 binding to cyclin B1.** IPs were performed with anti-cyclin B1 antibodies on extracts of mitotic cells, arrested in mitosis with Nocodazole after treatment with control shRNAi or arrested in mitosis by APC3-depletion. A control IP with rabbit antibodies was performed on extracts of the control mitotic cells. Whole cell extracts and IPs were blotted and probed with antibodies against the indicated proteins to analyse (co)-precipitation of these proteins. (E,F) **Cdc20 binds the APC/C independently of the APC3 subunit.** E) The same extracts as in D) were used to immuno-precipitate the APC/C with anti-Cdc20 antibodies. The IPs were blotted and probed with antibodies against the indicated proteins to detect (co-)precipitation of these proteins. Identical amounts of Cdc20 were precipitated and only a moderate reduction of bound APC4 was detected, revealing the ability of Cdc20 to bind remaining APC/C complexes after RNAi-depletion of APC3. F) Extracts of mitotic U2OS cells arrested in the spindle checkpoint or arrested after treatment with APC3-RNAi were used to immuno-precipitate the APC/C with anti-APC6 antibodies. The IPs were blotted and probed with antibodies against the indicated proteins to detect complexes. Cdc20 was still present in the APC6-IPs after APC3 depletion, although the Cdc20 levels were reduced compared to the control (compare lanes 3 and 4, middle panel). However, a similar reduction of APC2 was observed (lane 3 and 4, lower panel). Reduction of APC3 in the APC6-IP is much greater than the decrease of Cdc20 or APC2, so we concluded Cdc20 can still bind APC/C complexes that lack APC3.